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Toxicologie Analytique & Clinique

Volume 29, Issue 1,
Pages 1-146
February 2017

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Toxicologie Analytique & Clinique (2017) **29**, 1—2

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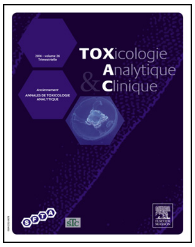

EDITORIAL

New psychoactive substances: Challenges of sharing analytical data and knowledge in a European regulatory context

This special issue ''*New psychoactive substances: analytical and clinical issues*'' including a number of reviews and original articles is particularly welcome because it allows the presentation of recent cases and new findings in this area where sharing of information and knowledge is becoming increasingly important for scientists and regulatory bodies active in the field, but also for policy makers and international organizations.[1]

New Psychoactive Substances (NPS) appeared on the European Drug market during the nineties. Over the past decade, an increasing number of NPS have been detected in Europe. The European Union established the Council Decision (EC) No. 2005/387/JHA on the information exchange, risk-assessment and control of new psychoactive substances. Within this regulatory framework, the EU Early Warning System allows the Member States to directly report data to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and Europol on events such as law enforcement seizures, acute poisonings and deaths. The analysis of data may then lead to public health alerts, law enforcement operations, risk assessments and, in some cases, control measures decided by the Council of the European Union.

An overview regarding the phenomenon of NPS in Europe can be found in the recent report ''*EU drug markets report: in-depth analysis*'' [1].

In the past two years, about two new substances were detected for the first time every week in Europe. The total number of new substances monitored in Europe will very likely reach more than 600 in 2016. This figure is more than twice the number of drugs controlled under international drug control conventions. Moreover the diversity of these substances is increasing. Seizure data from law enforcement agencies confirm the growth and importance of this drug market. Moreover, many of these new substances are vastly more potent than their controlled counterparts.

The observed trend and these figures are rather concerning and several action points have been identified to tackle the issues posed by the rapid emergence of NPS. For example, capacity of forensic science and customs laboratories to identify new substances should be increased. In order to protect public health, the strengthening of the EU Early

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[1] The United Nations General Assembly at the Special Session on the world drug problem (UNGASS, April 2016) adopted the outcome document ''Our joint commitment to effectively addressing and countering the world drug problem'' with a specific section about addressing emerging and persistent challenges and threats including NPS. The joint commitment calls for strengthening action to address the challenge of NPS as well as for enhancing information-sharing and early warning networks. http://www.unodc.org/postungass2016/

http://dx.doi.org/10.1016/j.toxac.2016.11.001

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Warning System and the national systems that support it will be required. Robust epidemiological indicators should be developed to allow the collection of reliable information on use of NPS across Europe.

In order to facilitate the collection of pertinent data at European and global level, the rapid access to the latest knowledge and analytical data should also be facilitated for the laboratories active in the front line for identification of new substances, for clinical treatment of cases of intoxication and also for forensic evidence of the use of NPS. Although strongly required, the sharing of analytical data, reference materials and expertise is more challenging than one may think at first glance, especially when considering the number and rapid evolution of the substances that are detected on the market. The unambiguous identification of a new substance requires the use of advanced analytical techniques such as high-field Nuclear Magnetic Resonance (NMR) and High Resolution Mass Spectrometry (HRMS). On the other hand, routine screening techniques are often based on gas chromatography hyphenated with low resolution mass spectrometry techniques and infrared spectrometry. In clinical studies, the detection of use of NPS is a further analytical challenge where identification of metabolites and/or the parent molecule in biofluids can be particularly tricky because of the low concentrations encountered for the more potent ones and the lack of knowledge about many of them. Thanks to its sensitivity, liquid-chomatography hyphenated with mass spectrometry is certainly one of the analytical techniques of choice for this area, either for quantitative purposes (e.g. with Triple Quadrupole instruments) or identification (e.g. with Time of Flight or Orbitrap instruments).

The collection of knowledge and the establishment and validation of analytical data on NPS (and possibly of their metabolites) with a broad range of analytical techniques will become increasingly required in the next future. This will be needed for the implementation of the proposed future EU legislation on NPS, which will establish a shorter time frame for risk assessment and decision-making [2]. Faster and more collection of data, and information too, especially for clinical data, will then be required for this process.

This special issue provides a useful overview of the current practice in the field.

Disclosure of interest

The author declares that he has no competing interest.

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Received 31 October 2016;
accepted 3 November 2016

Available online 30 November 2016

Toxicologie Analytique & Clinique (2017) **29**, 3

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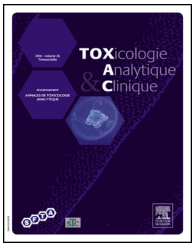

EDITORIAL

Chernobyl syndrome?

On April 26, 1986, massive explosions ripped through the Chernobyl Nuclear Power Plant. A ball of flame, with clouds of black smoke, rose into the sky. During several days, winds carried the radioactive cloud through the Soviet Union and Western Europe. In France, the Chernobyl disaster was marked by the so-called ''radioactive cloud that stopped at Strasbourg''. It is still debated that authorities had hidden the truth to the French population by denying that this radioactive cloud had really passed over the country and/or had not adequately informed the French population about the risks linked to the Chernobyl radioactive fallout.

NPS, also known as ''research chemicals'', ''bath salts'' or ''legal highs'', have appeared since 2000 and are mainly sold via Internet websites. Today, the global market for NPS expands and a growing number of NPS is reported each year throughout the world. In comparison with most of our neighboring European countries, there are relatively few studies and reports about NPS use in France: only a few ''French'' intoxication cases reported in the literature, almost no available epidemiological data about NPS use in the French population, a weak institutional communication about this emergent phenomenon, as well as relatively little information on NPS in the French media.

For a long time, I have been wondering if this situation was due to the absence of significant consumptions of NPS in France, and/or the inability of French laboratories of toxicology to detect NPS users, and/or a lack of interest from the French authorities to consider (and, consequently, to implement the necessary means to evaluate) potential and obvious problems related to NPS in France.

I still have not made up my mind on that question. However, I am sure that (1) NPS circulate and are experimented or regularly used in France (in an extent that has yet to be determined), and that (2), fortunately, some French laboratories of toxicology (especially ones collaborating together) are already able to detect efficiently NPS users. Most manuscripts in this issue successfully illustrate these two facts.

Disclosure of interest

The author declares that he has no competing interest.

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Received 20 October 2016;
accepted 21 October 2016
Available online 5 December 2016

http://dx.doi.org/10.1016/j.toxac.2016.10.003
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Toxicologie Analytique & Clinique (2017) **29**, 4—10

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REVIEW PAPER

Interpretation of NPS results in real hair samples

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Received 7 November 2016; received in revised form 23 December 2016; accepted 23 December 2016
Available online 17 January 2017

KEYWORDS
Hair;
New psychoactive substances;
Synthetic cannabinoids;
Synthetic cathinones;
Interpretation

Summary Today, the use of hair analysis for forensic issues concerning drugs collectively indicated as NPS is still controversial. In particular, little is known about the incorporation into the keratin matrix after intake and the correlation between their dosage, passive exposure, use frequency, and hair concentrations. In the present review, we considered the main issues, which still deserve substantial research and discussion within the scientific community, before a definitive interpretation of either a positive or negative results can be safely given to the local authorities. Specifically, the following scenarios were considered: (i) passive exposure vs. active consumption, (ii) mindful vs. unaware intake, and (iii) sporadic vs. chronic use. Differently from the traditional drugs of abuse, whose chemical and toxicological properties have been largely elucidated, in the context of NPS the range of chemical structures is so various that it is difficult to speculate about general criteria. Under these circumstances, any analytical outcome from NPS hair analysis should be cautiously interpreted by experienced forensic toxicologist.

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http://dx.doi.org/10.1016/j.toxac.2016.12.008
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Introduction

Around a decade ago, the first reports were published that described the existence of an unprecedented class of ''new psychoactive substances'' (NPS) distributed under the misleading name of ''legal highs'', ''designer drugs'', ''herbal highs'', ''bath salts'' or ''research chemicals'' [1,2]. Immediately after, forensic and clinical laboratories worldwide had to face the analytical challenge of the identification and quantification of these new drugs in various biological matrices. The commercial unavailability of reference standards for the parent drugs and their metabolites, the lack of updated and comprehensive immunoassays for their detection, and the extensive, yet insufficient, investigation of their metabolic transformation after intake, represented just the main problems toward the identification and quantification of NPS [3,4].

Nowadays, most of the aforementioned challenges have been overcome, at least partially. Several laboratories are offering screening and confirmation analysis for NPS in the context of workplace drug testing, driving re-licensing, roadside control and withdrawal programs. The identification of a certain NPS in urine and blood proves the recent exposure to this substance, enabling the authorities to prosecute and/or sanction the sample donor according to the local legislation. On the contrary, the use of hair analysis (in general, any keratin matrix) for forensic issues concerning NPS is still argued. The simple detection of NPS in hair has been extensively described in a recent book by one of the authors [3]. However, the results interpretation presents several controversial issues, as is quite common in hair analysis [3,5,6]. In the present review, we considered the main matters currently under debate, which still deserve substantial research and discussion before a definitive interpretation of either positive or negative results can be safely given. Specifically, the following alternative circumstances were considered: (i) passive exposure vs. active consumption, (ii) mindful vs. unaware intake, and (iii) sporadic vs. chronic use.

For consistency, the terms ''synthetic cathinones'' and ''synthetic cannabinoids'' were exclusively used. Drugs frequently classified under different categories or with different chemical structure were regrouped in an attempt to obtain comprehensive classification of similar drug subsets. Other groups of new designer drugs (e.g. opioids and designer benzodiazepines) were not included because their detection in hair has been reported only sporadically until today. Therefore, any comment about the interpretation would appear hasty.

Passive exposure vs. active intake

The most common administration routes for synthetic cathinones are insufflation (snorting) and oral ingestion of capsules or tablets, or powder wrapped in cigarette paper and swallowed (so-called ''bombing''). Quite often, the substance is dissolved or diluted with water/juice drink, to give an intoxicating beverage. Rectal insertion, intravenous, subcutaneous, and intramuscular injections are less frequently reported [7,8]. On the other hand, synthetic cannabinoids are predictably smoked. For both classes of NPS (synthetic cathinones and synthetic cannabinoids), external contamination is possible, especially when the drug is handled in relatively big amounts, as it may occur to drug dealers and police officers, so that contamination from hand contact or residues on furniture surfaces — like in homes formerly used as a clandestine drug laboratory — is likely and needs to be taken in consideration [9—15]. As for other biological matrices, the identification of metabolites is recommended also in hair analysis [16,17], as the only way for the exclusion of external contamination in most cases.

Synthetic cathinones

In 2012, Shah et al. developed a LC-MS/MS method for the quantitative analysis of mephedrone and two of its metabolites, namely 4-methylephedrine and 4-methylnorephedrine, in hair [18]. The authors screened the hair of 154 healthy volunteers for mephedrone, but only five samples tested positive, four of which at very low level. The metabolites were not detected (LOD: 5 pg/mg) in any of the analyzed samples while mephedrone could be successfully quantified in only one sample at a concentration of 21.1 pg/mg. In no case, it had been possible to confirm the positive result with the donor's admission of mephedrone use. The authors also noted that contamination from environmental exposure was not likely because mephedrone is not smoked.

In a recent paper [19], the concurrent detection of mephedrone, a mephedrone isomer (namely 3-methylmethcathinone) and two metabolites (3-methylephedrine and 3-methylnorephedrine) in pubic hair samples was obtained by means of liquid chromatography—high resolution/high accuracy Orbitrap mass spectrometry. In the presented case, a man was charged with dealing of NPS-containing materials. The powders and tablets seized by police contained 4-methylethcathinone (4-MEC), 3-methylmethcathinone (3-MMC), α-methylaminovalerophenone (pentedrone), 6-(2-aminopropyl) benzofuran (6-APB), 1-(benzofuran-5-yl)-N-methylpropan-2-amine (5-MAPB). The alleged drug dealer claimed that he was also a consumer of such drugs, and he intended to demonstrate his personal use for legal purposes. The pubic hair sample of the drug dealer turned out to be negative for 4-MEC, pentedrone, 6-APB, and 5-MAPB following LC-HRMS analysis. Conversely, 3-MMC was detected in pubic hair and quantified at a concentration of 25.8 ng/mg. The authors also sought to detect any metabolite of 3-MMC in the pubic hair sample of the drug dealer and actually identified 3-methylnorephedrine, 3-methylpseudonorephedrine, 3-methylephedrine and 3-methylpseudoephedrine. Their estimated concentrations were about one third (3-methylephedrines) and one thirtieth (3-methylnorephedrines) of 3-MMC concentration. This is consistent with the results usually observed in toxicological hair analysis, due to the lower incorporation of polar metabolites in the hair matrix with respect to the parent compounds. The metabolites' detection enabled to prove personal use of 3-MMC by the drug dealer, even though pubic hair contamination from urine cannot be completely excluded.

Synthetic cannabinoids

As for Δ^9-tetrahydrocannabinol, the mere detection of any parent synthetic cannabinoid in hair samples does not exclude the possibility of external contamination, nor does it provide conclusive evidence of active drug consumption. As a matter of fact, only the detection of their metabolites and possibly the evaluation of concentration ratio between parent drugs and metabolites can sustain the active use of synthetic cannabinoids and in most cases exclude external contamination from side-stream smoke or material handling.

The first studies to investigate the presence of NPS metabolites in hair were presented by Kim et al. [20,21]. In their first study [20], the authors validated an analytical method for simultaneous detection of JWH-018 and JWH-073, and their most abundant mono-hydroxylated and carboxylated metabolites. The method was applied to 18 hair samples from individuals suspected of using synthetic cannabinoids. Among the positive results, only the N-(5-hydroxypentyl) metabolite of JWH-018 (JWH-018 N-5-OH) was found, suggesting its prevalence in hair. Its concentrations were found to vary over a wide range, and the same was recorded for the ratio between parent drug and metabolite. Even some hair containing relatively high concentrations of JWH-018 (above 50 pg/mg), did not show the presence of JWH-018 N-5-OH. The highest concentration recorded for JWH-018 N-5-OH was 85 pg/mg, corresponding to a JWH-018 concentration of 151 pg/mg. Overall, in samples positive to both JWH-018 and JWH-018 N-5-OH, the parent drug-to-metabolite ratio was highly variable, ranging from 1.1 to 62.8. Noteworthy, JWH-018 N-5-OH is also the main product of AM-2201 metabolism [22], opening up the chance that particularly high concentrations of JWH-018 N-5-OH in hair may be generated by the concurrent ingestion of several NPS. In conclusion, the results of JWH-018 N-5-OH levels in hair are at the moment inconclusive and a comprehensive metabolite screening of the most popular synthetic cannabinoids, including AM-2201 appears to be necessary. In the second study of the same group [21], the method previously developed was extended to AM-2201, JWH-122, MAM-2201 and their mono-hydroxylated metabolites in hair. The method was also applied to investigate the distribution in authentic human hair samples taken from real forensic cases and the relative incorporation rate of AM-2201 and its metabolites in pigmented and non-pigmented rat hair. In real samples, JWH-018, JWH-018 N-5-OH, JWH-018 N-COOH, JWH-073, JWH-073 N-COOH, AM-2201, AM-2201 N-4-OH, AM-2201 N-6-OH indole, JWH-122, JWH-122 N-5-OH and MAM-2201 were detected, either simultaneously or individually. The concentration range of the parent drugs (e.g. AM-2201) was found to be much larger than that of the corresponding metabolites (e.g. JWH-018 N-5-OH). The parent synthetic cannabinoids and their mono-hydroxylated metabolites were identified in the hair samples of all nine cases, confirming that the simultaneous determination of both parent drug and metabolites in hair is helpful for most synthetic cannabinoids in order to exclude the possibility of passive contamination and also provide valuable information about the spectrum of molecules potentially ingested [21].

The issue of possible external contamination has also been raised by several authors [9,23,24]. In the first study [9,23], the extent of external contamination caused by handling drug material containing synthetic cannabinoids under realistic conditions was evaluated in a forensic laboratory. Hair samples from laboratory workers involved in the analysis of 670 herbal mixtures (covering 31 brands and 12 synthetic cannabinoids) within a two-weeks period were tested for synthetic cannabinoids with a validated LC-MS/MS method. In addition, hair samples from laboratory staff not working in direct contact with the drug material and close relatives of the exposed subjects were analyzed to check for cross contamination. All samples of workers in direct contact with the drug material tested positive for at least one synthetic cannabinoids. The measured concentrations ranged from trace level up to a maximum of 170 pg/mg (JWH-210) and roughly reflected the duration of exposure. Unexpectedly, some subjects not having direct contact to the drug material also showed measurable drug concentrations in hair. In one case, the JWH-210 concentration measured in the hair sample of a worker who was involved in the study was less than 0.5 pg/mg, whereas that detected in the hair sample of his girlfriend, who lived in the same household, but had no contact with the drug materials, was up to 11 pg/mg. Overall, the hair drug concentrations determined by mere external contamination were found in the range overlapping that typical for known drugs users, even if the majority of them was below 50 pg/mg. It was concluded that the actual consumption of synthetic cannabinoids can be unquestionably proved only by the detection of their metabolites in hair or, alternatively, the simultaneous positive testing of either urine, blood, or oral fluid.

In another study, the concentrations of synthetic cannabinoids were measured in scalp hair after exposure to side-stream smoke from a cigarette containing JWH-018, JWH-122, and JWH-210 [24]. The study showed that synthetic cannabinoids remain linked to the hair shaft long time after exposure to side-stream smoke. Since these substances cannot be completely removed by routine washing procedures prior to analysis, and the concentration ratio between wash solutions and hair extracts does not necessarily reflect external contamination, the positive results caused by side-stream smoke exposure can lead to erroneous conclusions.

The reliability of hair testing to reveal synthetic cannabinoid abuse was also questioned by Saito et al. [25], whose study proved that significant adsorption of synthetic cannabinoids occur in the hair of a non-user after passive exposure. The authors exposed both cosmetically treated and untreated scalp hair by soaking with NNEI and MAM-2201 aqueous solutions. The experiments showed that both compounds were partially adsorbed by both untreated black and dyed hair and were not easily eliminated by means of a regular washing procedure.

A further study [26] was aimed to develop and validate an analytical method for simultaneous detection of XLR-11 and its metabolites in hair. The method was applied to investigate the distribution of XLR-11 and its metabolites in 14 authentic human hair samples: XLR-11, UR-144, UR-144 N-5-OH, UR-144 N-COOH, and XLR-11 N-4-OH were detected with widely variable distributions of their quantitative results.

Recently, the first results from a comprehensive screening of drugs and metabolites on a large group of subjects were presented [27] and later expanded to a larger population [3]. Overall, 23 samples were tested positive for synthetic cannabinoids. In 16 cases, low concentrations of the parent drug (below 50 pg/mg) were measured and no metabolites were found. For these cases, sporadic exposure represents the most likely event, even if external contamination could not be excluded. In four cases, samples were tested positive for multiple drugs at relatively high concentrations (above 50 pg/mg), but negative for metabolites. Frequent exposure to synthetic cannabinoids is the most likely explanation, even though no metabolites were found. The remaining records included three samples that exhibited very high concentrations for different synthetic cannabinoids and also some metabolites at very low concentration. The latter cases are fully compliant with frequent exposure to synthetic cannabinoids, proven by (i) high levels, (ii) multiple positive testing, and (iii) presence of metabolites.

It is not unrealistic that certain known NPS metabolites can be formed also by different routes, not involving metabolic transformations. To evaluate several aspects possibly affecting hair analysis results for synthetic cannabinoids, the stability of 5F-PB-22 and AB-CHMINACA was assessed by analyzing smoke condensates and hair samples stored under different conditions [28]. For comparison, an authentic hair sample of a patient with known history of heavy synthetic cannabinoids consumption was analyzed in segments. In all of them, 5F-PB-22 and AB-CHMINACA were detected, together with three metabolites: 5F-PB-22 3-carboxyindole, PB-22 5-OH-pentyl, and AB-CHMINACA valine. Alternative origins of these substances was investigated in smoke condensates and contaminated hair (by 5F-PB-22 and AB-CHMINACA) after thermal stress. In smoke condensates, both 5F-PB-22 3-carboxyindole and AB-CHMINACA valine were detected. This experiment proved that both products can be formed as pyrolytic artifacts during smoking, and indicated side-stream smoke as a possible source of contamination for these alleged metabolites. On the other hand, the thermally treated contaminated hair samples also showed the presence of 5F-PB-22 3-carboxyindole, PB-22 5-OH-pentyl, and AB-CHMINACA valine, along with their respective parent compounds. Again, it was demonstrated that both drugs undergo hydrolysis after deposition onto the hair, preventing clear distinction between true metabolites and artifacts.

Mindful vs. unaware intake

Quite remarkably, the majority of the published studies describing NPS detection in real hair samples, reported the frequent occurrence of poly-abuse [29—33]. Actually, no systematic correspondence exists between herbal blend trade name and real content in synthetic cannabinoids, making the consumers rarely aware of the actual composition of the purchased products. This is not surprising, because herbal blends are not standardized products, but rather semi-clandestine preparations obtained from mixtures of herbal leaves of different origin, on which various synthetic cannabinoids are sprayed. According to the cannabinoids availability, the active ingredients may vary from lot to lot, even when the trade name is the same. It was also shown that cannabinoids concentration may vary significantly among different packages of the same brand [34—37] and that some blends may contain two or more active compounds [35,38—40]. Variable amounts and combinations of these ingredients are therefore put together in ''spice'' products to generate cannabis-like effects. It is not uncommon that further substances with similar or different pharmacological activity are added to herbal mixtures [3,41]. This inconstancy of the ''legal highs'' contents implicate that consumers do not have control the power and the effects of the product they are consuming [38].

Likewise, it has been shown that pills or powders purchased as stimulants drugs often contain more substances than is declared on the label [7,35]. Doubts about drug identity, purity, actual dosage, multiplicity, and potential synergistic effects are fundamental unanswered questions that expose NPS users to hazardous consequences [35]. Jang et al. [42] reported a fatal poisoning involving param-ethoxyamphetamine (PMA). Upon hair analysis, PMA was detected at a concentration of 20.1 ng/mg, suggesting consumption of this drug in the two months before death. Ketamine and MDMA were also detected at high concentrations in the same specimen. The authors speculated that the deceased drug user might have ingested PMA expecting to be taking MDMA, as it occurred in many other PMA-related fatal cases.

An innovative approach to gain information on NPS diffusion combined questionnaire administration and hair sampling from nightclub/festival-attending young adults (age 18—25) in New York City [43]. Out of participants who accepted to donate an hair sample, 48 also reported habitual use of ecstasy/MDMA/Molly. Half (50.0%) of hair samples actually contained MDMA, 23 contained butylone (47.9%), and 5 methylone (10.4%). Of those who reported no lifetime use of any ''bath salts'', stimulant NPS, or unknown pills or powders, 41.2% tested positive for butylone, methylone, alpha-PVP, 5/6-APB, or 4-FA, suggesting that many ecstasy-users among nightclub/festival attendees may unintentionally purchase ''bath salts'' or other NPS in place of MDMA. In another study [44], 23 real samples taken from proven MDMA and ketamine abusers were tested for the presence of 31 NPS, among cathinones and other stimulant, psychedelic and dissociative designer drugs. Some NPS were detected in multiple samples, in particular methoxetamine (3 samples, range of concentration: 7.7—27 pg/mg), mephedrone (2 samples, respectively 50 and 59 pg/mg), while other drugs were identified in a single sample: 4-MEC (330 pg/mg), methylone (< LOQ), α-PVP (1040 pg/mg), 4-FA (55 pg/mg), MDPV (120 pg/mg) and diphenidine (4400 pg/mg), proving past poly-abuse of several NPS.

Sporadic vs. chronic use

The range of chemical structures collectively indicated as NPS is so various that it is difficult to determine or even speculate about the binding capacity of each single substance to the keratin matrix. On the other hand, the pharmacological potency of NPS tested *in vitro* is generally extremely high, and it is believed that these compounds are extremely active at relatively low doses also *in vivo*. The low dosage at which high-potency NPS are taken reduces the chance to find detectable levels in hair even under the favorable circumstance that the drug has high affinity toward the keratin matrix. Lack of knowledge about both effective dosage of NPS and their keratin binding make any attempt to establish a reasonable cut-off value for each drug questionable. Some laboratories set the limit of detection as the minimum hair concentration to ascertain the use of NPS, but this practice does not settle the matter if cut-off values should be used in hair analysis in order to discriminate between chronic consumption and occasional use (or even single scouting intake). At the moment, only few papers dealt with the expected NPS concentration in real samples, and even less studies have investigated the relation occurring between detected levels and frequency of use.

One of the first papers that reported the detection of NPS in hair [45] found thirteen samples positive for mephedrone, with relatively high concentrations (range: 0.2—313.2 ng/mg, mean: 26.8 ng/mg). The authors concluded that mephedrone is likely to be extensively incorporated into hair, like other stimulant drugs such as amphetamines or cocaine.

In a case of a 25-year-old man found dead in the apartment of a friend [46], hair analysis revealed past exposure to mephedrone (0.25 ng/mg). This finding was consistent with the statement of the decedent's friend, reporting that he was a consumer of new designer drugs.

Wikström et al. [47] described two fatal intoxications with the new designer drug methedrone (4-methoxymethcathinone). In the first case, hair was not sampled during the autopsy. In the second case, short hair segments (three 5-mm and two 10-mm segments) revealed an even distribution of methedrone (segment one: 37 ng/mg, segment two: 33 ng/mg, segment three: 29 ng/mg, segment four: 29 ng/mg, and segment five: 36 ng/mg), suggesting chronic intake of methedrone over the months preceding the intoxication.

A method for the detection of 4-MEC and MDPV was validated and applied to a 30-year-old man who usually consumed cathinones for 6 months administered intravenously [48]. Both 4-MEC (30 ng/mg) and MDPV (1 ng/mg) were identified in the hair at high concentrations showing a regular consumption of these drugs.

The detection of the dissociative anesthetic designer drug diphenidine in hair was recently described. In a case of a first time use [49], a hair sample was obtained 49 days after ingestion, and was divided into five 1 cm segments. Diphenidine was identified on the first three proximal segments, probably due to sliding hair in the strand, at concentrations of 123, 79 and 89 pg/mg.

In another case, diphenidine was detected at 4400 pg/mg concentration in the hair sample of a 30-year-old Caucasian man with previous history of drug addiction [50]. According to the authors, the high hair concentration indicates that the subject had previously been exposed to diphenidine in several occasions.

An interesting discussion about the incorporation of NPS in hair was proposed by Namera et al. [51], in a report dealing with the detection of pyrrolidinophenone-type designer drugs (such as MDPV and α-PVP) in patients suspected to consume illegal drugs. The authors speculated that pyrrolidinophenone-type designer drugs should be expected to have a low incorporation rate into the keratin matrix because of the beta-carbonyl group in their structure. In contrast, α-PVP was found in several segments from different subjects, with the correspondent highest hair concentrations ranging from 10 to 300 ng/mg. They concluded that the positive binding effect of the pyrrolidine group due to its hydrophobic character might exceed the negative effect of the beta-carbonyl group. Also MDPV was detected in this study as well as in another report from the same authors [52], at even higher concentrations, in which hair samples were collected from a 35-year-old woman found unconscious and pronounced dead at the hospital. Segmental analysis was conducted to confirm chronic drug abuse. MDPV and α-PVP were detected in ten and five consecutive 10-mm segments, respectively, with the highest MDPV concentration found at 22 ng/10-mm hair. Analogous investigations about the rate of incorporation of NPS were proposed by Nieddu et al. [53], who evaluated the different accumulation of some target phenethylamines occurring between pigmented and non-pigmented rat hair. Concentrations above 0.20 ng/mg were detected only in pigmented hair.

Also for synthetic cannabinoids, several studies dealt with real samples from forensic casework but only rarely the analytical determination was completed with information about the frequency of past use. Hutter et al. [30] obtained hair samples from a population of forensic psychiatry patients who admitted chronic consumption of several herbal mixtures in the last few months before sampling. The self-stated intake frequency ranged from three times during six weeks up to daily consumption of half a package for seven months. Eight samples tested positive for several synthetic cannabinoids, with concentrations ranging from 0.5 to 78 pg/mg. Other studies presented real hair sample analysis [29,31—33,54,55], yielding a broad range of concentrations encompassing 3—4 orders of magnitude but no accessory information about past use. From these studies, it was hardly possible to draw any conclusion about the possible correlation between the NPS use history and the concentrations measured in hair. Although the cut-off level of 50 pg/mg is internationally accepted for THC in hair, there are no enough data in current literature to draft similar conclusion for synthetic cannabinoids. Further studies should be performed to discriminate between sporadic and chronic use, particularly when the concentrations detected are lower than 50 pg/mg [29].

Conclusions

For several decades, the studies concerning drugs of abuse were restricted to a very limited number of substances, whose chemical and toxicological properties were progressively elucidated. In contrast, hundreds of NPS came to prominence in the last decade, making it impossible to determine for each one the chemical, pharmacological, and toxicological properties, together with their biological distribution and metabolic fate. Unfortunately, even less is known about their incorporation into the keratin matrix after intake and the correlation between their dosage, use frequency, and hair concentrations. Under these circumstances, any possible conclusion that a forensic toxicologist may draw from hair analysis about NPS abuse have high chance to be seriously questioned in court, particularly when the detected hair concentrations are lower than 50-100 pg/mg. For this reason, it is important (i) to interpret hair testing data about NPS with extreme care, (ii) to use published studies whenever possible, but keeping any conclusion strictly connected with the context under examination, (iii) to develop original, updated and thorough investigations on the properties of the most abused NPS in relation with their effects on hair testing. Such investigations are nowadays facilitated by the large availability of adequate analytical instrumentation and validated methods, which are extremely sensitive and accurate at the same time.

From the studies published up to now, a few preliminary conclusions can be tentatively outlined. First, the detection of NPS metabolites appears to be an extremely valuable means to sustain active use and to differentiate active use from passive intake. However, one should be aware that, for some synthetic cannabinoids, alleged metabolites can occasionally be produced also from non-metabolic processes and therefore be detectable in hair even after external contamination. Secondly, the limited data still available from both chronic and sporadic NPS users do not allow to estimate the frequency of use from hair concentrations. However, within homogeneous populations and conditions, large differences in hair concentrations can be interpreted as an effect of use frequency. Third, the scenario of NPS present on the black market is totally unpredictable at any time, in terms of quality and quantity of active principles contained in the finished product. Therefore, it is inappropriate to infer any user's addiction features for NPS from the analytical outcome of hair analysis alone, especially when low concentration levels are detected. As a matter of fact, unaware intake of NPS, sold as surrogates of more traditional drugs, appears to be recurrent, yielding unprecedented hazards of intolerance, adverse effects, and fatal overdose.

Disclosure of interest

The authors declare that they have no competing interest.

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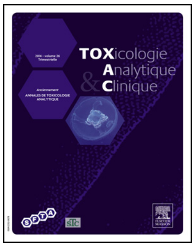

REVIEW PAPER

Challenges concerning new psychoactive substance detection in oral fluid

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Received 21 November 2016; received in revised form 13 December 2016; accepted 13 December 2016
Available online 12 January 2017

KEYWORDS
New psychoactive substances;
Oral fluid;
Sampling protocol;
Detection;
Judicial challenges

Summary New psychoactive substances (NPS) are a challenge for the worldwide forensic community. More than 450 NPS are currently being monitored by the UNODC and EMCDDA. NPS are a major challenge for laboratories, not only because of the large numbers of NPS, but also due to the continuous chemical development of the substances and thus the rapid turnover of the drug market. The aims of our publication are to give an overview of practical considerations when applying oral fluid (OF) analysis in the context of NPS detection, to highlight the current limitations and to address options to improve current research and legislative actions. OF sample collection and protocols will be discussed as differences will have an impact on the final obtained concentrations via dilution, drug (in)stability and recovery issues. In addition, the current state and the future possibilities for NPS detection in OF using direct assays or chromatographic techniques are described. Finally, judicial challenges and applications are discussed.

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http://dx.doi.org/10.1016/j.toxac.2016.12.004
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Introduction

New psychoactive substances (NPS) are a challenge for the worldwide forensic community. A total of 541 NPS was reported to the early warning advisory of the United Nations Office on Drugs and Crime (UNODC) [1] by 95 countries up to December 2014. In the European Union (EU) 450 of these substances are currently being monitored [2]. NPS are defined by the EU Council Decision 2005/387/JHA as a psychotropic drug in pure form or in a preparation that has not been scheduled under the 1961 or in the 1971 United Nations Single Convention on Narcotic Drugs and that may pose a threat to public health comparable to the substances listed in either schedule I, II, III or IV [3]. They can be newly synthesized/designed compounds, but they can also be research chemicals, nutritional substances or (failed) medications that are now commercialized in the form of bath salts, plant food or incense. These substances include synthetic cannabinoids [4], cathinones [5], tryptamines, designer stimulants and designer medication (opioids, benzodiazepines, etc). Some of the NPS are still considered as ''legal highs'' and are largely distributed via e-commerce, although several countries have developed either listed or generic legislations to counter the spreading of NPS and their potential health risks.

NPS are a major challenge for laboratories, not only because of the large numbers of NPS, but also due to the continuous chemical development of the substances and thus the rapid turnover of the drug market. This makes it difficult for the laboratories to have up-to-date methods for NPS monitoring in bulk form or in biological samples. While classical drugs of abuse still dominate the global drug market, there seems to be an increase in the use of NPS. According to the US National Survey of Drug Use and Health (2009—2013) the lifetime prevalence of NPS use in 12 to 34 year olds was 1.2% [6]. In the EU, youngsters with an age between 15 and 24 years ($n = 13{,}000$) reported their use in 2014 via a telephone based assessment: 8% reported to have used NPS, 1% in the last 30 days, 3% in the last 12 months and 4% more than a year ago [7]. In blood samples obtained in a Driving under the influence of Drugs (DUID) setting after a positive on-site oral fluid test (Druwipe-5S®, Securetec, Germany) a prevalence of 5% was observed [8]. When analyzing oral fluid (OF) obtained from overdose patients ($n = 55$) treated at the Oslo Accident and Emergency Outpatient Clinic, 13% of the cases were found positive for a NPS [9]. Even though NPS prevalence for specific groups or settings is described in literature [6—10], we should be aware that these are estimates, not only because of the study design, but also because detection techniques have to be updated in order to get a broad picture of the abused NPS types. Moreover, the impact of other factors such as the degree of metabolism or the (in)stability of these compounds in biological matrices on the final analytical result or detectability are not yet extensively examined.

One of the biological matrices that are of interest to detect NPS is OF. OF has been considered as a valuable toxicological matrix in the past decade certainly in fields were high-throughput is required (e.g. the fields of DUID and in workplace drug testing) [11]. The main reason of its growing success is the non-invasiveness of the collection which can be done by non-medical trained personnel in a rather easy way ensuring the privacy of the tested subject. In addition, because the free fraction of parent compounds is detected in relatively high concentrations, this matrix is of interest to prove recent drug use. In two recent reviews, the NPS classes as well as chromatographic methods for their detection in OF were thoroughly discussed and summarized [10,12]. Therefore, the aims of our publication are to give an overview of practical considerations when applying OF analysis in the context of NPS detection, to highlight the current limitations and to address options to improve current research and legislative actions.

NPS deposition and pharmacokinetics in OF

Several reviews are published discussing the pharmacokinetics of NPS [13—15]. Due to ethical reasons, controlled in vivo studies are usually not available, resulting in limited information concerning the disposition of NPS (and their metabolites) in OF as well as their detection windows. Drugs are transported from the blood into OF in the salivary glands either by passive diffusion through the cell membranes, ultrafiltration through the membrane pores or via active transport via receptors. The type as well as the degree of transport depends on the physicochemical properties of the drug (pKa, lipid solubility, molecular weight, and spatial configuration), the degree of plasma protein binding, and the pH ratio of blood and OF. Passive diffusion is by far the most common mechanism of drug transport, and is limited to non-protein bound, non-ionized molecules, with a molecular weight of less than 500 Da and a certain degree of lipophilicity. OF, will thus theoretically predominantly contain the parent drug due to the higher lipid solubility [16]. The way a drug is consumed, however, has an influence on the final OF concentration as shortly after drug use by smoking, oral ingestion or nasal insufflation, contamination of the oral cavity can lead to dramatically elevated OF concentrations of the parent drug. NPS are used via various routes of administration (e.g. oral, sublingual, inhalation, injection). Kneisel et al. reported an OF concentration of 0.02 ng/mL of the synthetic cannabinoid (SC) AM-2201 after oral intake of 5 mg in a gelatin capsule prepared to avoid oral contamination. This result indicates that SC are excreted from salivary glands only at a very low rate. However, after smoking (three pulls from) a self-made joint containing different types of SC, concentrations ranged from 7 to 577 ng/mL depending on the volunteer and the substance, indicating a deposition of the substances in the oral cavity. The concentrations dropped by 70%, 30 minutes after smoking and the SC were detectible at least for 6 hours up to 55 hours [17]. Coulter et al. reported peak concentrations of 3—35 ng/mL, 20 minutes after smoking herbal blends containing JWH-018 with detection windows of 5—12 h (when applying a cut-off of 0.5 ng/mL) for OF samples collected with the Quantisal™ device [18]. More research concerning the disposition of NPS in OF should be established. In addition, the occurrence of passive contamination and the necessity of detection markers (e.g. metabolites) focusing on active consumption should be investigated.

Sample collection and sampling protocol

When applying OF as a toxicological matrix it all starts with the collection of the sample. OF collection can be achieved via spitting, drooling or collection via various types of absorbent swabs. OF collectors are preferred because of hygienic reasons and because these contain stabilizing buffers which insure proper analysis. However, the choice of the OF collector has to be made wisely as it will have an impact on the final qualitative and quantitative result, due to their effect on drug stability, drug recovery and the dilution ratio (amount of OF collected versus amount of stabilizing buffer). Not only the OF collectors will have an influence on the sampling procedure, but also other parameters in the sampling protocol such as the storage conditions of the final sample (e.g. storage time, temperature and type of container [polypropylene, glass, etc.]) [19,20].

(In)stability data of NPS in neat as well as diluted OF from different collectors are scarce. In most publications the validation includes processed sample stability, but long-term stability is often not investigated. Kneisel et al. [21] did examine the stability of 11 SC in neat OF in glass or polypropylene containers for 24 to 72 h at 4 °C or 25 °C. No instability was observed after storage in glass containers, however, in polypropylene tubes a significant loss was observed after 24 h at 25 °C for most SC, while at 4 °C only JWH-251 seemed unstable. According to Rodrigues et al. [22], 40% of the original SC concentrations could be detected in OF when collected in the Quantisal™ (Immunalysis) collector and stored at room temperature for 7 days. After 6 weeks only 20% remained. When stored refrigerated, 50—60% remained intact after 7 days, but only 25—35% after 8 weeks. For long-term storage, this research group suggested the samples should be frozen. Miller and Concheiro [23] evaluated the stability of 10 synthetic cathinones (cathinone, methcathinone, buphedrone, mephedrone, 4-methylethcathinone (MEC), 3,4-methylene-dioxypyrovalerone (MDPV), methylone and naphyrone, alpha-pyrrolidinovalerophenone (PVP), and N-ethylcathinone) in OF collected via the Quantisal™ collector. They concluded that synthetic cathinones are stable at 4 °C and −20 °C for one week and after 3 freeze-thaw cycles. Naphyrone, methylone, MDPV and mephedrone, however, were not stable at room temperature for neither one week nor 24 h as losses up to 45% were observed. These studies are of immense importance as these data are critical for re-analysis of samples for counter-expertise or for retrospective analysis of NPS. Hence, more of this type of investigations including different types of NPS, but also for other types of diluted OF, and long-term storage conditions are necessary.

The literature reports adsorption of especially lipophilic compounds to the collection swab and plastic surfaces of the sampling device of classical drugs of abuse [19,24]. This loss in recovery will probably also be observed for the lipophilic NPS. Kneisel et al. [25] observed a difference in SC recovery from the Dräger DCD5000 collector depending on the storage time and chemical structure. Apparently SC with a hydrophilic moiety such as a morpholino or an N-methylpiperidine group showed better recoveries than other SC. The best recoveries were obtained after 3 days of storage at room temperature with addition of 1 mL ethanol. Rodrigues et al. [22] observed that the recovery of SC from the Quantisal™ collector was not concentration dependent and the lowest observed recovery was 68% for JWH-018. Again, more research should be done to evaluate the effect of recovery on the detection ability of NPS in OF samples.

One of the drawbacks of OF collection is the limited sample volume which can be obtained, certainly in subjects suffering from dry mouth caused by stress, smoking and drug use [11]. This collected amount of neat OF will even be further diluted when collected by an OF collector with stabilizing buffer. Gjerde et al. [26], however, observed that the prevalence of drugs found in OF samples with a small volume was higher than in those with a large volume indicating that the obtained low volume is not necessarily problematic. Drug concentrations tend to decrease with an increasing salivary flow because of a dilution effect and a pH increase due to the changed OF bicarbonate concentration. This will be the case for most NPS, — with the exception of SC — as they are often basic, lipophilic compounds with a comparatively low molecular weight. Non-stimulated collection can thus be advantageous, however, if (unstimulated) sampling takes too long, this will not be practical in certain situations e.g. OF collection for a drugged driving population.

To conclude, collection differences will have an impact on the final concentrations via dilution, instability and recovery issues. The impact of the OF collection on the obtained concentrations should hence be carefully investigated. As a result published OF concentrations of NPS observed in case reports should always be evaluated in light of collection step and caution has to be taken when comparing data from different case reports or databases for toxicological interpretation.

Immunological and chromatographic detection

The major problem for the detection of NPS is the continuous chemical development of NPS entering the market at a high pace and with a rapid turn over time. This has an impact on the development of detection techniques (immunological as well as chromatographic) as they have to be developed in such a way that they enable an up-to-date detection. In general, we observe that toxicologists all over the world state that the detection of NPS in first instance — and not their quantification — is of utmost importance [27]. The first priority of the forensic community needs to be the detection of every NPS for a good estimation of their prevalence and possible impact in a forensic case, before we can even start establishing the degree of effects and toxicity of these compounds.

A problem seems to be that currently applied immunoassays focusing on ''classical'' drugs of abuse have insufficient cross-reactivity for NPS. In addition, not a lot of immunoassays are tested or developed for detection in OF. Nieddu et al. [28] evaluated the cross-reactivity of two OF immunological screening platforms, the Screen® Multi-Drug (Screen Italia Srl, Perugia, Italy) and the GIMA One Step Multi-Line Screen Test (GIMA Spa, Gessate, Milan, Italy), for 39 new amphetamine-like designer drugs. Most drugs and/or NPS had very low cross-reactivity, except for p-methoxyamphetamine (25%—50%) and p-methoxymethamphetamine (25—250%) which are the p-substituted analogues of amphetamine and methamphetamine, leading to the conclusion that the developed OF screening platforms are very selective towards their targets and not to NPS. Recently, the same group investigated the cross-reactivity of 30 new amphetamine designer drugs, using the amphetamine and methamphetamine/MDMA specific Neogen® test [29]. Concentrations of 10,000 ng/mL were not sufficient to produce a positive response for the majority of the analyzed amphetamines, clearly demonstrating that these tests will not reliably identify NPS in real casework as these concentrations greatly exceed those expected to be found in forensic samples [29]. For the SC cross-reactivity from immunoassays targeting natural cannabinoids is expected to be negligible as they are structurally quite different. Therefore, dedicated immunoassays could be a possible answer. Several commercially available immunoassays have already been marketed by Randox Technologies and Immunalysis. However, the immunoassays focusing on specific SC (or other NPS) can be quickly outdated as they target a chemical structure and because the NPS market evolves quickly. Development of high-throughput screening techniques focusing on their activity, rather than chemical structure therefore seems to be the future [30]. A proof of concept is already demonstrated by Cannaert et al. via the detection of in vitro cannabinoid receptor activation by SC (receptors CB1 and 2) and their metabolites in urine using a newly developed G-protein coupled receptor activation assay based on the NanoLuc® Binary technology (Promega) [30]. The further development of this technique may result in a relatively simple, non-radioactive and sensitive first-line screening tool for SC (and other NPS), complementing targeted and untargeted analytical assays and preceding analytical confirmation techniques.

The chromatographic detection and quantification methods for NPS in OF are described in several recent reviews [10,12]. The challenges for method development concerning the detection of NPS in OF are to make the methods sensitive enough to detect a lot of compounds in a small sample volume and to keep them up-to-date. In addition, during the method development and validation the analyst should always keep in mind the demands of proper validation, as required by accreditation agencies concerning qualitative or quantitative purposes, but also practical issues when applying a certain analytical technique e.g. thermolability if applying gas chromatographic methods. The first choice to make is a targeted (specific) versus non-targeted (more general) method. Targeted methods often use Multiple Reaction Monitoring (MRM) to obtain very sensitive methods. This might be necessary certainly as the main drawback of OF collection is the limited sample volume [11], which will even be further diluted depending on the selected collector. Specific target methods, such as the ones described in the recent literature [10,12], can thus be of interest, however, sometimes the NPS described are already outdated. These methods need continuous adaptation via constant introduction of MRMs of new NPS standards. Obtaining these reference materials (including metabolites) is difficult as they are not always commercially available and therefore pose a restriction for method development. An option is to buy a NPS from the internet to use as a standard, however, the laboratory always has to elucidate the chemical structure first via various spectroscopic, mass spectrometric and chemical methods. Another point to make is that maintaining a reference standard library for compounds that are only encountered either briefly or infrequently is not feasible for most laboratories due to constraints on costs and resources. Therefore, the international community is establishing projects such as RESPONSE sponsored by the EU to ensure up-to-date NPS libraries including mass spectral and fourier transform infrared (FTIR) data, and availability of reference materials hoping to diminish the cost and workflow for laboratories and customs [31]. Non-targeted general screening procedures, however, can offer a solution as even if one's library is not up-to-date, a retrospective analysis is always possible. Advanced techniques involving high-resolution mass spectrometry offer the advantage of a possible elucidation of chemical structures via accurate mass in a sensitive way. Several workflows for detection of NPS in biological matrices using time-of-flight are already published and could be applied to OF [32,33]. Of course, when more general methods are applied, the sample preparation should also be general and not exclude compounds. As a result, the current development of ambient ionization techniques such as desorption electrospray ionization (DESI) or direct analysis in real time (DART) could potentially be of interest to ensure elucidation of unknown structures without demanding multistep sample preparation. This technique has already been evaluated for SC in herbal blends, powders and biological matrices, but to our knowledge not yet in OF [34]. Adequate sensitivity of this technique for the detection of NPS in OF has yet to be proven. In the current literature concerning detection of NPS in OF, classical extraction techniques such as solid phase or liquid/liquid extraction, precipitation and/or dilution steps are applied [10,11]. The dilute-and shoot techniques will not exclude compounds, but, matrix effects should always be carefully evaluated. Matrix effects will not only occur due to the differences in the OF matrix because of circadian rhythm, etc. but are also due to the stabilizing buffers in the OF collection kits which contain salts, non-ionic surfactants and antibacterial agents. One of the newer ideas to tackle matrix effects obtained from stabilizing buffers, without tampering stability, is the application of dried OF spots. Lødøen et al. [35] examined the application of 10 μL OF to alginate and chitosan foams obtaining OF dried spots containing methadone, methamphetamine, buprenorphine, para-methoxyamphetamine and para-methoxymethamphetamine. However, before this technique can take its place in a forensic toxicological setting, the practical and legal issues but also the feasibility in a context where a lot of NPS should be analyzed in this limited volume should be investigated extensively.

Judicial issues and applications

Currently, legal responses are developed worldwide to tackle the NPS problem. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) summarized these legislative developments in a number of reports [2,36—38]. Broadly speaking, three types of legal responses could be distinguished. In the first group, existing laws concerning consumer safety, health protection, or medicines have been used to stop the open distribution of NPS. Examples are the Netherlands, Finland and Austria [1,39,40]. The second group concerns existing drug laws that have been adapted, most commonly by introducing generic legislation (group definitions of substances under control). Several countries have introduced group definitions, such as ''synthetic cathinones'' in Cyprus, France, Italy, Lithuania, Norway, United Kingdom; ''synthetic cannabinoids'' in France, Cyprus, Italy, Lithuania, Luxembourg and Norway; ''tryptamines'' in Lithuania and Norway and ''phenethylamines'' in Cyprus, Lithuania and Norway [36]. The third group covers so called ''innovative'' new laws that have been designed by different countries, such as the United Kingdom, Ireland, Sweden, Portugal and Austria [36,38].

Three main aspects of the innovative legal responses can be identified: (a) the criteria used to define the substance or the motivation for use, in a few cases even defining a substance by its psychoactive effect rather than its chemical structure; (b) the listing mechanisms for reducing the time needed to control new substances and (c) the levels of punishment [38]. Some countries have initiated more than one response, either simultaneously or consecutively. Belgium opted for a generic legislation, which will shortly be operational once the Royal Decrees are published. This legislation makes a broad range of NPS illegal and implies monitoring of these illegal substances in bulk form by customs or police, but also in biological samples by clinical and forensic toxicological laboratories for judicial and public health concerns. In other terms, organizations such as forensic toxicological laboratories will have to be able to monitor these compounds in a direct and up-to-date way and legislations focusing on drug use (e.g. drugs and driving legislations) should be adapted if the legislator wants to focus on these substances too.

We strongly believe that OF is an interesting matrix in the context of medico-legal casework focusing on living human beings. OF is an attractive matrix for confirmation in the laboratory as it can be easily obtained on-site even by non-medically trained personnel without hampering privacy of the test subject. Due to the fact that the parent compounds will be predominantly found, it is easier to include and detect new compounds [41]. However, only time can tell if additional metabolite detection will be of importance for interpretation issues. Although OF drug concentrations cannot be used to directly estimate blood concentrations and finally their effect because of for example contamination of the oral cavity just after smoking or oral intake of a drug, the presence of a NPS points to recent drug use. In addition, OF drug detection appears to be superior to urine in correlating positive findings with impairment symptoms [42]. Thus, NPS detection in OF will be useful for certain legislations focusing on drug use (and not related to effect) such as per se DUID legislations, or in the fields of workplace drug testing and rehabilitation institutes. Of course, as laboratory resources are becoming more restricted, a pragmatic approach for the determination of NPS has to be established taking into account the aim of the legislation/approach, the costs and the prevalence of NPS in a specific target group. In other judicial casework such as Drug Facilitated Crimes (DFC) or post-mortem cases, the detection of NPS will be necessary if the full picture of the case has to be elucidated. However, in those settings, OF is not considered to be the ideal matrix, due to its detection window (DFC) or impossibility of collection (post-mortem).

At the moment, NPS cannot be detected on-site due to the lacking cross-reactivity of on-site screening panels and the difficulty of keeping up with the continuous developing drug market. Thus, while developments in the redesign of such on-site tests are going on, legislators and laboratories will have to focus on detection in the laboratory with chromatographic techniques. For DUID detection, an initial OF on-site screening for classical drugs is often applied, followed by a blood confirmation or OF confirmation [43]. Because of the risk that drivers under the influence of NPS (or other not screened drugs) will not be detected on-site, the legislator should foresee an option for the law enforcement to obtain an OF sample for confirmation if clear signs of impairment are observed even though a negative on-site test is produced. The way the OF sample is collected will thereby play a major role, because the choice of OF collector will have an impact on the final result, which was already explained earlier. Therefore, the legislator should fix the collection system and protocol to ensure that the whole legislative area works under the same conditions; the chain of custody, the collection protocol (stimulated OF collection or not and time-frame), transportation conditions (focusing on stability), and sample handling in the laboratory should be fixed. Another challenge is the interpretation of the test result. As OF concentrations cannot be correlated to effects, the question arises concerning the applied cut-offs or reference values. To date, no international cut-off values are established for NPS, because no danger cut-offs (neither in blood nor OF) have already been established. The main issues in determining suitable cut-offs are the following: individual variations, drug tolerance, interactions with other drugs, and the variable effects of the same oral fluid concentrations of drugs depending on whether the concentration is rising or falling. Since all these things are largely unknown for most NPS, setting legal limits is difficult to establish. As a result, Gerostamoulos et al. [27] proposed to currently focus on qualitative determination of NPS for routine forensic purposes. In this view, Bogusz [44] proposed the use of ''minimum required performance limit'' (MRPL) values, which refer to a minimum content of an analyte in a sample, which at least has to be detected and confirmed. Use of MRPLs intends to harmonize the analytical performance of methods for substances for which no permitted limit has been established. Hence, this harmonization will lead to the benefit of an equal quality of analysis and it may provide results that are used in a uniform way in court. Other points of concern in the procedures are to obtain enough sample to ensure adequate analysis and a possible counter-expertise, meaning also that the analyzing laboratory has to perform the initial testing on a small volume using highly-sensitive equipment. It is necessary to analyze the same sample as the repeatability of two consecutively taken OF samples is not guaranteed [45]. Concerning counter-expertise, the question also remains if all laboratories will be able to assess all new regulated NPS. Moreover, one of the advantages of non-targeted screening is the option of retrospective data-analysis. However, challenge will be how to reconcile this retrospective analysis in a legal context? For example in a

DUID or DFC case we can ask ourselves what do we do if a retrospective analysis demonstrates the use of a NPS once the court has already established a definitive jurisprudence.

Conclusion and future perspectives

Current literature has proven that the detection of NPS in OF is feasible and of interest in a forensic toxicological setting. The major challenge for NPS is the current change in the drug market and thus the growing concern that the prevalence of NPS in forensic casework is underestimated. Toxicology laboratories should therefore be encouraged to develop their methods to detect a broader range of drugs beyond the standard panel of illicit drugs, providing an overall view of NPS. Until this is regular practice, the availability, use but also the degree of effects and toxicity of NPS will remain unknown.

OF is often criticized as it can only target recent drug use and it does not establish a link to possible effects. This, however, is to date also the case for NPS in blood, as no reference values are set yet. OF can thus offer a simple, directly observable and non-invasive sample collection with an adequate NPS detection. However, there are still a lot of challenges to be addressed and research to be conducted. First of all, the impact of the sampling of OF and storage protocols should be investigated more thoroughly. In addition, toxicological laboratories should adapt their workflow and analytical methods. Considering costs and laboratory management aspects, it is imperative to invest in method development and data exchange, in close collaboration with other parties or laboratories. Moreover, international organizations could help out by creating adequate NPS libraries, making (legislative) changes to ensure easy access to reference materials, offering guidelines for sampling protocols and minimum requirements concerning the necessary analytical methodologies including thresholds or MRPL. Once these issues are addressed NPS detection in OF will have a major impact on forensic casework in the fields of DUID and DFC, and for workplace drug testing or rehabilitation institutes.

Disclosure of interest

The authors declare that they have no competing interest.

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Toxicologie Analytique & Clinique (2017) **29**, 18—33

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ORIGINAL ARTICLE

A 6-year review of new psychoactive substances at the *Centre antipoison Grand-Ouest d'Angers*: Clinical and biological data

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Received 29 November 2016; received in revised form 7 December 2016; accepted 7 December 2016
Available online 4 January 2017

KEYWORDS
Designer drugs;
New psychoactive substances;
NPS;
Cathinones;
Acute exposure

Summary For the last 10 years, recreational use of new psychoactive substances (NPS) with hallucinogenic properties was more and more described either in a context of consumption during a rave-party or at home alone or in small group. A review of 62 cases (56 M, 6 F, 16—46 y) registered from 2011 to 2016 at the French Grand Ouest centre antipoison of Angers is presented. Some of them ($n = 15$) presented a severe clinical state (poison severity score, PSS = 3) and only one was a fatal intoxication (PSS = 4). Treatment of acute intoxication was mainly based on supportive care with a quick recovery in less than 48 h. A high number of these cases were documented by plasma (pl) and/or urine analyses using LC-UV, GC-MS and LC-MS^2. Several powders or other forms bought on the web market by the patients were also analysed. Six amphetamines derivates were detected such as methiopropamine (pl: 50 — 430 µg/L, $n = 6$) or 5 others (6-APB, 6-APDB, 5-EAPB, 5-MAPB, 2-FA each once). Four 2,5-dimethoxy-derivates of phenylethylamines (2Cs) or of amphetamines (DOs) were detected as 2C-P (pl: < 10—20 µg/L, $n = 5$), 2C-T-4 (88 µg/L) or 2C-E (only in one urine) and DOC ($n = 5$, pl: < 10—18 µg/L). In 24 cases,

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http://dx.doi.org/10.1016/j.toxac.2016.12.001
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12 different cathinones were observed in plasma samples (*n* = 8) as MDPV (pl: 10—40 μg/L, *n* = 4), 3-MMC (pl: 7—300 μg/L, *n* = 6), 4-MEC (pl: 240 — 890 μg/L, *n* = 3), mexedrone (pl: 100 and 180 μg/L) and methylone (3130 μg/L in a peripheral post-mortem blood). Others cathinones (4-MMC, α-PVP, 4-chloro-α-PVP, bk-2C-B, MDPP, MPHP, pentedrone) were essentially observed in urine or in powders. Two tryptamine analogs were found as AMT (pl: 440 μg/L in one plasma sample) and 5-MeO-DALT (*n* = 5). The 3 ketamine-like compounds (*n* = 17) detected in plasma were methoxetamine (pl: 8.6—360 μg/L, *n* = 12), methoxphenidine (pl: 254—590 μg/L, *n* = 3) and diphenidine (pl: 20 μg/L, *n* = 1). Five other miscelleanous drugs were found in 11 cases and 9 different synthetic cannabinoids (AB-CHMINACA, AB-FUBINACA, AKB48, 5F-AKB48, 5FUB-AKB48, 5F-PB22, AM1220, MDMB-CHMICA, UR-144) were found in 7 different patients. For the same period, illicit amphetamines constituted 52 cases with amphetamine or ecstasy intake sometimes combined with NPS. These data were presented to show the increasing use of wide-range NPS in a French region and to provide quantitative data in clinical situations besides the large information on the post-mortem cases available in the literature.

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Introduction

For the last 10 years, recreational use of new psychoactive substances (NPS) with hallucinogenic properties was more and more described either after consumption during a rave-party or at home alone or in small group. The easy buy via the Internet and the ''legal'' status of more of them are in favour of increasing consumption and represent a large potential risk to public health. The EMCDDA—Europol annual report notified 98 new substances described for the first time in 2015 while only 40 described in 2010 and 365 different NPS detected as seizures across Europe in 2014 [1]. As described by Peters and Martinez-Ramirez [2], Maurer [3] and Tyrkkö et al. [4], the two main classes of the so-called designer drugs or ''legal highs'', are the large cathinone family and the synthetic cannabinoids, but other non-exclusive chemical classes are represented by the phenylethylamines or amphetamines derivatives and their 2,5-dimethoxy analogues (2Cs and DOs families) or the methylendioxy- compounds (ecstasy analogs), the benzofuran derivatives (''benzofuries''), the piperazine derivatives, the alkyl tryptamines and the ketamine analogues. Many articles reported exposures to these different classes of substances often as fatal case reports but sometimes as nonfatal exposure reports. Today, the survey of such consumption represents a challenge for the clinical emergency units [5] and the toxicological laboratories.

We present here a review of the cases registered from 2011 to 2016 at the French Grand Ouest centre antipoison of Angers. Some of them were previously described as original papers [6—10] or only mentioned in different abstracts following oral presentations or posters [11—21].

Material and methods

A retrospective study listed all the calls received by the western France poison control centre (PCC) at Angers university hospital that were related to exposures to NPS. The western France poison centre usually receive notifications regarding a large proportion of poisoning cases that occur in four French regions, including a population of more than 12 million people. A total of 58,000 calls are received each year, and the proportion of calls from physicians equals 56% (vs. 39% from the general public and 5% from nurses and paramedics). Cases between January 2011 and October 2016 were reviewed. Data related to phone consultations involving Angers PCC were extracted from the Poison control centre database, as authorized by the French data protection authority (Commission nationale informatique et libertés [CNIL], accreditation no. 747735). This database was supplemented by reports from clinical staff in hospitals. The personal data of patients were anonymized before their medical records were studied. Approval of the local Ethics Committee was unnecessary given the retrospective and non-interventional design of this study. The severity of poisoning was assessed using the Poison Severity Score (PSS), according to the following grades: none (grade 0), minor (grade 1), moderate (grade 2), severe and fatal (grade 3—4) poisoning [22]. The overall PSS retrospectively evaluated the severity of poisoning once the final outcome was known. This score was developed and tested with respect to both functional and lesion-causing toxicants, including medicinal products, and was validated by a prospective, multicentre study. The final PSS score was based on the most severe symptoms of poisoning. Clinical symptoms were then evaluated throughout hospitalization. Only cases with analytical data were included.

The analytical identification and quantification of the suspected compounds were performed using different analytical approaches as drug of abuse immunoassays and chromatographic processes: gas chromatography coupled to mass spectrometry (GC-MS), high performance liquid chromatography using diode array UV detection (LC-UV/DAD) or/and tandem mass spectrometry (LC-MS^2). Plasma and/or urine samples were analysed in addition to powders obtained from patients upon admission to an intensive care unit (ICU). Purification of biological samples was performed by two different liquid-liquid extraction processes (one under alkaline pH conditions and the other one under initial alkaline condition followed by a back-acidic extraction) for the LC-UV process coupled with spectral identification with a home-made library containing more than 1000 spectra. For the LC-2D-MS^2, a simple protein precipitation was used [10]. To 100 µL plasma or diluted urine was added 150 µL methanolic solution containing methylmilnacipran (200 µg/L) as internal standard (IS). The supernatant was further diluted 4-fold in water before chromatography (injection 10 µL). On-line column concentration was performed on a Strata X (25 µm, 20 × 2 mm) with ammonium formate 2 mM (flow 2 mL/min) and, after 1 min, elution was done on a Kinetex phenyl-hexyl column (2.6 µm, 100 × 3 mm, Phenomenex, Le Pecq, France) under gradient solvent conditions (0.6 mL/min) as previously described. MS^2 detection was performed in positive electrospray ionization mode (ESI+) and with multiple reaction monitoring (MRM, two transitions per compound). Several compounds such as cathinones, were identified using GC-MS after chemical derivatization with heptafluorobutyric anhydride [8]. Powders or Web samples were solved or extracted in analytical grade methanol before chromatographic analyses. Reference pure substances were obtained from LGC Standards (Molsheim, France).

Results

Sixty-two patients (56 M, 6 F, 16—46 years, median 31 y) were included between January 2011 and October 2016. Geographical repartition covered 20 French departments. All clinical and toxicological data were presented in Table 1. Fig. 1 showed the frequency of the different detected substances and their distribution per year. The number of cases increased from 2011 to 2015 with 4, 9, 11, 12 and 19 patients respectively. Only 7 cases were documented in 2016.

Amphetamine compounds

NPS of this family are essentially represented by methiopropamine (MPA), a thiophen analog of metamphetamine, always in a context of polyconsumption (pl: 50 — 430 µg/L; ur: 12.6 to 37.6 mg/L, *n* = 6). This compound presented a good response with some amphetamine urine immunoassays [23]. Other compounds (*n* = 5), such as aminopropylbenzofurans (APBs), aminopropyldihydrobenzofurans (APDBs) or fluoroamphetamine were also observed. During the same period, methylenedioxymetamphetamine (MDMA), its N-demethylated metabolite (MDA) (*n* = 27) and amphetamine (AM) (*n* = 24) were the main illicit compounds while only one case involved methylenedioxyethylamphetamine (MDEA) and, surprisingly, no metamphetamine was documented using toxicological screening over this 6-year period.

2Cs and DOs analogs

Among the eleven 2Cs analysed in the lab, only 3 were observed in this study. In five male patients (17—22 years), 2,5-dimethoxy-4-(n)-propylphenethylamine (2C-P) was detected in all urine samples (range: 40—1670 µg/L) but quantified only in one plasma (20 µg/L) while this substance was detected below LOQ (10 µg/L) in the other 4 cases [9,11]. Furthermore, a study of human metabolism was performed on the urine samples by GC/MS, LC/MS^n and LC-high-resolution-MS^n and compared with metabolism in rats [24]. 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4) was detected in one case, instead of the suspected ethylthio-analogous substance (2C-T-2), and this case is described more in details below. Between March 2014 and March 2015, five male patients (16—23 years) were admitted in an emergency unit after consumption of drugs either at a rave-party (*n* = 3) or in a private (*n* = 2) context. A white powder, named ''DOC'', was also obtained in one case and allowed the identification of suspected ingested drug as 2,5-dimethoxy-4-chloroamphetamine (DOC) [20,25]. Plasma levels ranged from 10 to 18 µg/L in 3 cases but were below 10 µg/L (LOQ) in the 2 others while urine levels (*n* = 5) ranged from 300 to 1300 µg/L.

Cathinone family

Mephedrone or 4-methylmetcathinone (4-MMC) was detected in one multi-component powder while it has never been detected over the 6-year period in plasma or urine samples. One acute exposure to the homologous ethyl compound (4-MEC) administered intravenously and combined with gamma-butyrolactone (GBL) was published in 2014 [8,15]. Plasma levels (8 h after injection) were respectively 353 µg/L and 300 mg/L for MEC and γ-hydroxybutyric acid (GHB), the active metabolite of GBL. An additional study of the 4-MEC metabolism was performed with pooled liver microsomes and compared with the patient urine [26]. In two other patients, MEC plasma levels were 240 and 890 µg/L with 60.9 and 85 mg/L in urine. In one case, only the metabolites of 4-MEC were detected both in plasma and urine. Over the last 2 years, 3-MMC, the positional isomer of mephedrone, was detected in plasma (*n* = 5) or blood (*n* = 1) with concentrations from 7 to 300 µg/L and in only one available urine sample (41.6 mg/L). Moreover, in one case, 3-MMC was combined with GBL intake and GHB levels were 200 and 685 mg/L in plasma and urine, respectively. Mexedrone (MEX), the methoxy analog of 4-MMC, was found in 2 patients (pl: 10 and 180 µg/L; ur: 750 and 930 µg/L) and pentedrone was observed once (pl: 35 µg/L, ur: 1.1 mg/L). One fatal-intoxication after methylone ingestion was registered in the CAP database and described by another team with a 6640 µg/L cardiac blood level [27]. Methylone was detected in another patient combined with a pyrrolidinocathinone, methylenedioxyphenylpropiophenone (MDPP), with urine levels of 50 and 7.5 mg/L, respectively and also low levels of MDMA and MDA. Methylenedioxypyrovalerone (MDPV) was our first NPS observed in 2011 with a co-methoxetamine ingestion [6,13]. The MDPV plasma levels ranged from 10 to 40 µg/L in documented cases (*n* = 4). The MDPV level in urine, only obtained in the first case, was 2.3 mg/L. Another homologous compound, α-pyrrolidinovalerophenone (α-PVP), was detected in two urine samples (11.9 and 15.8 mg/L) as oxo-PVP, one of its metabolites (0.58 and 0.91 mg/L) [21]. Only this metabolite was detected (57 µg/L) in the single available plasma sample. However, another peak was observed in all plasma and urine samples that might be the hydroxy- metabolite

Table 1 Description of the 62 cases observed between January 2011 and October 2016.

Sex/Age	Suspected products/ Route	Clinical observations	PSS	[C] plasma μg/L	[C] urine μg/L	Powder	Ref.
M/32	Desoxypipradrol (2-DPMP) / PO	Agitation, audio and visual hallucinations, mydriasis	2	2-DPMP 136	2-DPMP 4805	N/A	[7,12]
M/17	2C-P / PO	Agitation, confusion, visual hallucinations, logorrhea, echolalia, tachycardia	2	2C-P << 5	2C-P 40	N/A	[9,11]
M/21	2C-P powder / sniff	Agitation, flushes, amnesia, mydriasis, sweating	2	2C-P 5	2C-P 330	2C-P 100%	[9,11]
M/32	MDPV/MXE / sniff	Palpitations, thoracic pain, agitation, vasoconstriction, tachycardia	2	MDPV 45; MXE 122	MDPV 2300; MXE 2950	N/A	[6,13]
F/36	‘‘pentedrone’’, MXE / PO	Hallucinations, mydriasis, QTc 500 ms, tachycardia, GS 3	3	MXE 200	MXE 26,630	4-MEC 100%	[6,14]
F/31	‘‘pentedrone’’, MXE / PO	Hallucinations, choreoathetosic movements, QTc 480 ms, tachycardia, GS 8	3	MXE 360	MXE 165,300	4-MEC 100%	[6,14]
M/39	‘‘pentedrone’’, MXE	Dysarthria	1	MXE 150	N/A	4-MEC 100%	[6,14]
M/38	‘‘pentedrone’’, MXE	Agitation, hallucinations, nystagmus	1	MXE 140	MXE 16,200	4-MEC 100%	[6,14]
M/43	‘‘NRG’’ MDPV? / IV	Comatose 2h after admission, GS 3	3	MDPV 8; Oxazepam 2700	MDPV 272; Oxazepam	N/A	[6]
M/21	Methylone	Cardiorespiratory arrest, seizures, hyperthermia	4	Methylone 3130 (peripheral blood)	Methylone 5 10^5	N/A	[14]
M/40	2C-T2 20 mg? / sniff	Agitation, seizures, hallucinations, mydriasis; HTA	3	2C-T4 88	2C-T4 2400	N/A	[16]
M/23	MXE / PO	Confusion, mydriasis, GS 7	2	MXE 346	MXE 22,050	N/A	[6]
M/39	4-MEC, GBL / IV	Unconscious, transient apnea, confused for 4 h	2	MEC 300; GHB 3 10^5	MEC 10^5; GHB 10^6	4-MEC 100%	[8,15]

Table 1 *(Continued)*

Sex/Age	Suspected products/ Route	Clinical observations	PSS	[C] plasma µg/L	[C] urine µg/L	Powder	Ref.
M/21	2C-P + MXE?	Agitation, confusion, visual hallucinations, mydriasis, tachycardia	2	2C-P <<5; MXE 8.6	2C-P 347; MXE 1970	N/A	[6,9]
M/21	''mescaline'' / PO 9 drops	Hallucinations, delirium, mydriasis, tachycardia	2	2C-P 8	2C-P 1670	N/A	[9]
M/22	''mescaline'' / PO 4 drops	Confusion, disorientation, visual hallucinations, sweating, mydriasis, tachycardia	2	2C-P 20	2C-P 1160	N/A	[9]
M/33	6APDB, MDPV, 2FA, Etizolam / PO + powder MXE?	Unconsciousness, agitation, mydriasis, GS 10	2	MXE 24; MDPV 40; 2FA 400; 6-APDB 550; Etizolam?	N/A	MXE > 95%	[16]
F/41	6-APB / PO	Seizures, drowsiness, mydriasis, tachycardia	3	6-APB 200; Citalopram 60; Lamotrigine	N/A	N/A	[16]
M/30	MXE powder	Hallucinations, dissociative syndrome	2	MXE 680	MXE 90	MXE 60%	
M/30	Methadone	Hypotension, tachycardia, inverted Tako-Tsubo cardiomyopathy	3	AM 21; Methadone 180	AM 5200; Methadone 5500; Venlafaxine 1000	AM + caffeine	
M/46	NRG3 / sniff	Sweatness, paresthesia, anxiety	2	4MEC 200; MPA 180; MeODALT 25;MetPhe 100	4MEC 18,000; MPA 37,600; MeODalt 30; MetPhe 8000	PEA; MPA; Caffeine; 4MMC; 4MEC; MetPhe; MeODalt	[17]
M/26	Powders (*n* = 2)	Hypertonia, coma, seizures, hypersalivation, mydriasis, GS 4	3	MPA 50; MeODALT 30	MPA 1600; MeODALT 1600	MPA+ benzocaine; nicotine + menthol	[16,17]
M/33	Powder Bk-2C-B	Hallucinations, drowsiness, headache, mydriasis	1	N/A	Bk-2CB metabolite; Amprenavir	Bk-2C-B > 90%	
F/23	Halucium tablet (2C-E)	Palpitations, thoracic pain, mydriasis	1	2C-E not detected; Tramadol < 100	2C-E 29; Tramadol 300	2C-E 30 mg/tablet	

Table 1 (*Continued*)

Sex/Age	Suspected products/ Route	Clinical observations	PSS	[C] plasma µg/L	[C] urine µg/L	Powder	Ref.
M/37	Powders ($n = 4$) MXE, MXP, MPA, 5-FUR144 Liquid ($n = 1$)	Hallucinations, tremor	2	MPA 180; MXP 254; MXE 117; UR144 7[a]	MPA 22,600; MXP 4940; MXE 13,800; Citalopram 2000; Cyamemazine 200	MPA; MXE; MXP; UR144; MetPhe; MeODALT; 5F-AKB48	[17]
M/21	LSD blotter ? Ecstasy / PO	Agitation, hallucinations, mydriasis	2	DOC 13	DOC 720; MDMA 90; MDA 20	N/A	[20,25]
M/18	LSD blotter? Ecstasy / PO	Agitation, hallucinations, mydriasis	2	DOC 10; MDMA 190; MDA 14	DOC 1300; MDMA 50,000; MDA 2200	N/A	[20,25]
M/26	Diphenidine powder / IV	Agitation, logorrhea, mydriasis, tachycardia	2	DiPhe 200; MPA 390; Methadone 140	N/A	Diphenidine > 95%	
M/44	Powders ($n = 4$) D2PM/DPH?; 5EAPB; 5MeODALT; etizolam	Hallucinations, agitation, confusion	2	Hydroxyzine 140; Oxazepam 700; NDM Cyamemazine 30	N/A	Diphenhydramine; MeODALT; 5-EAPB; Etizolam	[17]
M/35	Et-Phenidate	Transient motor deficit	2	Et-Phenidate 25	Et-Phenidate 850	N/A	
M/30	Powders ($n = 4$) S1-S4	Hallucinations, dysarthria, hyperthermia, tachycardia	2	MDPV 30; MXE 150; Methadone 260; APB?	N/A	S1: MDPV + MXE; S2: MPA; S3: AM1220; S4: Etizolam	[19]
M/23	LSD? Ketamine, cocaine?	Agitation, visual hallucinations	2	DOC < 10; Ketamine 100; NK 250; BZE 175	DOC 320; K 9000; NK 7000; BZE 16,500	N/A	[20,25]
M/38	Unknown powder	Agitation, tonic-clonic seizures, hypersudation, mydriasis, tachycardia	2	Alpha-PVP < LOQ; Oxo-PVP 57	Alpha-PVP 11,880; Oxo-PVP 568	Alpha-PVP 100%	[21]
M/34	Et-phenidate (NATRIUM)?	Profuse sudation, hyperthermia, mydriasis: serotoninergic syndrome, tachycardia	2	Methadone 160;5F-AKB48 7[a]+ metabolites?	Methadone; EDDP; olanzapine; 5F-AKB48 metabolites?	5F-AKB48	[19]

Table 1 (*Continued*)

Sex/Age	Suspected products/Route	Clinical observations	PSS	[C] plasma μg/L	[C] urine μg/L	Powder	Ref.
M/21	Laughin Assassin, Buddha Blues, Walter White, Green Vapes	Visual hallucinations, mydriasis, tachycardia	2	5F-AKB48 1[a]; 5F-PB22 < 1[a]	SC not detected	BB: 5F-AKB48; WW: MPA; LA: 5F-PB22; GV: 5F-AKB48, Glycerine, nicotine	[19]
M/33	Yellow tablet: MXP? / PO	Hypertonia, hypersudation, mydriasis, tachycardia, GS 10, serotoninergic syndrome	3	AMT 440; methadone 300; lormetazepam 200	AMT 78,300; methadone 1300	AMT 30 mg/tablet	[10]
M/28	4-MEC?	Mydriasis, tachycardia	2	4MEC <LOQ (met++)	4MEC <LOQ (met++)	N/A	
M/39	Et-Phenidate	Agitation, hypertonia, mydriasis	1	Et-Phenidate 700	Et-Phenidate 7200	Et-Phenidate 45% + caffeine, lidocaine	
M/43	Methylone?	Delirium	2	N/A	Methylone 50,000; MDPP 7500; MDMA 310; MDA 65	Methylone 77% + MDPP 17%	
M/16	Amphet, LSD blotter?	Hallucinations, anxiety, mydriasis	2	DOC << 10 (2?)	DOC 300	N/A	[20,25]
M/23	Shyana shop Natrium	Hypertension, tachycardia, chest pain	2	Et-Phenidate 100	N/A	N/A	
M/21	''DOC''	Seizures, hyperthermia, mydriasis, tachycardia	3	DOC 18; MPA 430; Pentedrone 35	DOC 470; AMT 500; methadone 220; MPA 12,600; pentedrone 1120	DOC > 97%	[20,25]
F/22	3-MMC, 5MAPB, AB-Fubinaca?	Confusion, somnolence, myosis, GS 10	2	3MMC 300; 5MAPB 20; méthadone 100	N/A	N/A	[21]
F/22	MDPV?	Agitation, visual hallucinations, mydriasis	2	N/A	Alpha-PVP 15,840; oxo-PVP 910	N/A	[21]
M/38	5F-PB22, AB-Fubinaca	Acute respiratory detress syndrome, inhalation pneumonia, GS 5	3	MPA 410; venlafaxine 160; olanzapine 40	MPA 34,000; venlafaxine 15,000; olanzapine	5F-PB22 (100%) AB-Fubinaca (50%)	
M/35	3-MMC / IV	Agitation, hypertension, chest pain,tachycardia	2	3MMC 7	3MMC 2370	N/A	[21]

Table 1 (*Continued*)

Sex/Age	Suspected products/ Route	Clinical observations	PSS	[C] plasma µg/L	[C] urine µg/L	Powder	Ref.
M/37	Pandora, MJ Sweet	Confusion	1	MDMB-Chmica 12[a]; 5F-AKB48 1[a]	MDMB Chmica not detected	Pandora: 5F-PB22, MDMB-Chmica, 5F-AKB48? MJSweet: 5F-AKB48?	
M/42	Hawaiian baby woodrose seeds	Hallucinations, headache, vomiting	1	N/A	Not tested	Lysergic amide (LSA)	
M/24	Halo Purity: SC ? / PO	Headache, abdominal pain	1	No toxic identified	N/A	Nicotine, 1,2 Propylene glycol, No SC identified	
M/27	MDMB CHMICA	Agitation, logorrhea, euphoria, tachycardia	1	MDMB Chmica 5[a]	MDMB Chmica not detected	N/A	
M/23	LSD blotter? DOC?	Hallucinations, anxiety	1	BZE 200	BZE 50,000; cocaine; levamisole	LSD	
M/29	3-MMC	Hallucinations, tachycardia	2	3MMC 220 (whole blood)	N/A	N/A	[21]
M/32	Spice?	Anxiety, headache	1	AKB-48 2[a]	Cannabis ++	N/A	
M/38	Et-Phenidate	Hallucinations, hyperthermia, tachycardia	2	Et-Phenidate 1900; loxapine 40	Et-Phenidate 120,000; loxapine	N/A	
M/27	Salvia?	Hypertonia, obnubilation, mydriasis	2	THC 6,5; THC-COOH 77; AB-Chminaca > 25[a]; 5F-AKB48, FUB-AKB48 1-2[a]	AB-Chminaca < 1 + 5F-AKB48 metabolites?	AB-Chminaca, 5F-AKB48, FUB-AKB48	
M/31	Diphenidine?	Agitation, GS 10	2	MXP 590; loxapine 70	MXP 4800; oxycodone 130	N/A	
M/23	Powders (*n* = 4) 4-MEC, MXE?	Seizures, GS 3	3	3MMC 1600; 4MEC 890; MXE 1180	3MMC 141,000; 4MEC 61,000; MXE 39,000	3MMC 100%, 4MEC 100%, MXE 99% and 92%	
M/37	Powders (*n* = 2) MEX?	Agitation, mydriasis, tachycardia	2	3MMC 60; 4MEC 240; MEX 10; MDMA 110	3MMC 13,000; 4MEC 85,000; MEX 930; MDMA 5600; MDA 320	3MMC 3%/MEX 67%; 3MMC 70%/4MEC 30%	

Table 1 (*Continued*)

Sex/Age	Suspected products/ Route	Clinical observations	PSS	[C] plasma μg/L	[C] urine μg/L	Powder	Ref.
M/38	MXP; ? / parachuting	Agitation, hyperthermia, logorrhea, HTA 195/110, hallucinations, tachycardia	3	MXP 580; MEX 180	MXP 4100; MEX 750; venlafaxine 400	N/A	
M/38	Powders (4) identification request	N/A	—	N/A	N/A	4-Cl-a-PVP (94%); MPHP (100%, $n=2$); 5-MeODALT (91%)	
M/37	Stilnox, cocaine, New opiates?	Seizures 10 min after sniffing, myosis, hypoventilation, tachycardia, GS 4	3	Zolpidem 100; BZE 170; cocaine	BZE 25,000; cocaine; codeine 68; morphine < 10; zolpidem	Meta- or para-fluorofentanyl in mannitol	
M/30	Methadone, GBL, opiates	Coma, myosis, bradypnea, GS 3-5	3	3MMC 150; GHB 200,000	3MMC 41,600; GHB 685,000	N/A	

N/A: not available; PO: oral route; IV: intravenous route; others: see in text.

[a] Estimated concentrations of the detected synthetic cannabinoids.

formed by reduction of the keto function either *in vivo* or *in vitro*. Recently, 3 powder samples obtained in a forensic context, were analysed and identified as α-chloro-PVP ($n=1$) and 4-methyl-α-pyrrolidinohexanophenone (MPHP) ($n=2$). For this case, no biological samples had been obtained. Bk-2C-B, the keto-analog of 2,5-dimethoxy-4-bromophenethylamine (2C-B) was identified in one powder but the plasma and urine of the patient do not contain this compound but a substance with UV spectrum identical to 2C-B or to 2,5-dimethoxy-4-bromoamphetamine (DOB) but with a different retention time. This compound suspected to be the hydroxy-metabolite as described for the others cathinones was further confirmed by Maurer (data not published). In the different cathinone cases, re-analysis of samples stored at +4 °C showed a high decrease of plasma levels. So, when a case of cathinone exposure is suspected and if the quantification is not rapidly performed, long-term sample storage should be performed at −20 °C.

Indole tryptamine derivates

Alpha-methyltryptamine (AMT) was encountered in one plasma sample (440 μg/L) with a severe associated serotoninergic syndrome [10,18] and in one urine sample of DOC cases. The isomer of AMT, 5-(2-aminopropyl)-indole, was not observed in this study. 5-methoxy-N,N-diallyl-tryptamine (5MeO-DALT) was detected in 4 different situations. For the first patient, plasma level was 200 μg/L approximately 4 hours after consumption of a ''NRG3'' orange powder containing 6 other substances (phenylethylamine, MPA, caffeine, 4-MMC, 4-MEC, methylphenidate). Another case was documented with a concentration of 30 and 1600 μg/L in plasma and urine, respectively. MPA was also detected. In a third case, 5-MeO-DALT was identified in green pills (150 mg weight) found at home of a patient admitted 8 days before in a ICU for supposed ingestion of cocaine and etizolam (also identified in pink pills). Unfortunately, no blood samples were obtained. In the last case, 5-MeO-DALT was identified in a liquid (few μL) called ''NZT-48'', containing 5 other molecules (MPA, caffeine, cocaine, phenacetine and a synthetic cannabinoid further identified as 5F-AKB48—see below). LSD, not considered as NPS, was identified in a blotter paper (about 100 μg) but not quantified in the biological samples of the corresponding patient that contained benzoylecgonine (BZE), cocaine and levamisole, a usual adulterant. LSD was also searched but not found in some suspected cases and, in these cases, the detected molecule was DOC [25].

Ketamine-like compounds

Methoxetamine (MXE) was observed in 12 cases (8 previously described) despite its illegal status in France since the end of 2012 [6,13,14]. Levels ranged from 8.6 to 360 μg/L and from 0.09 to 165.3 mg/L for plasma ($n=12$) and urine

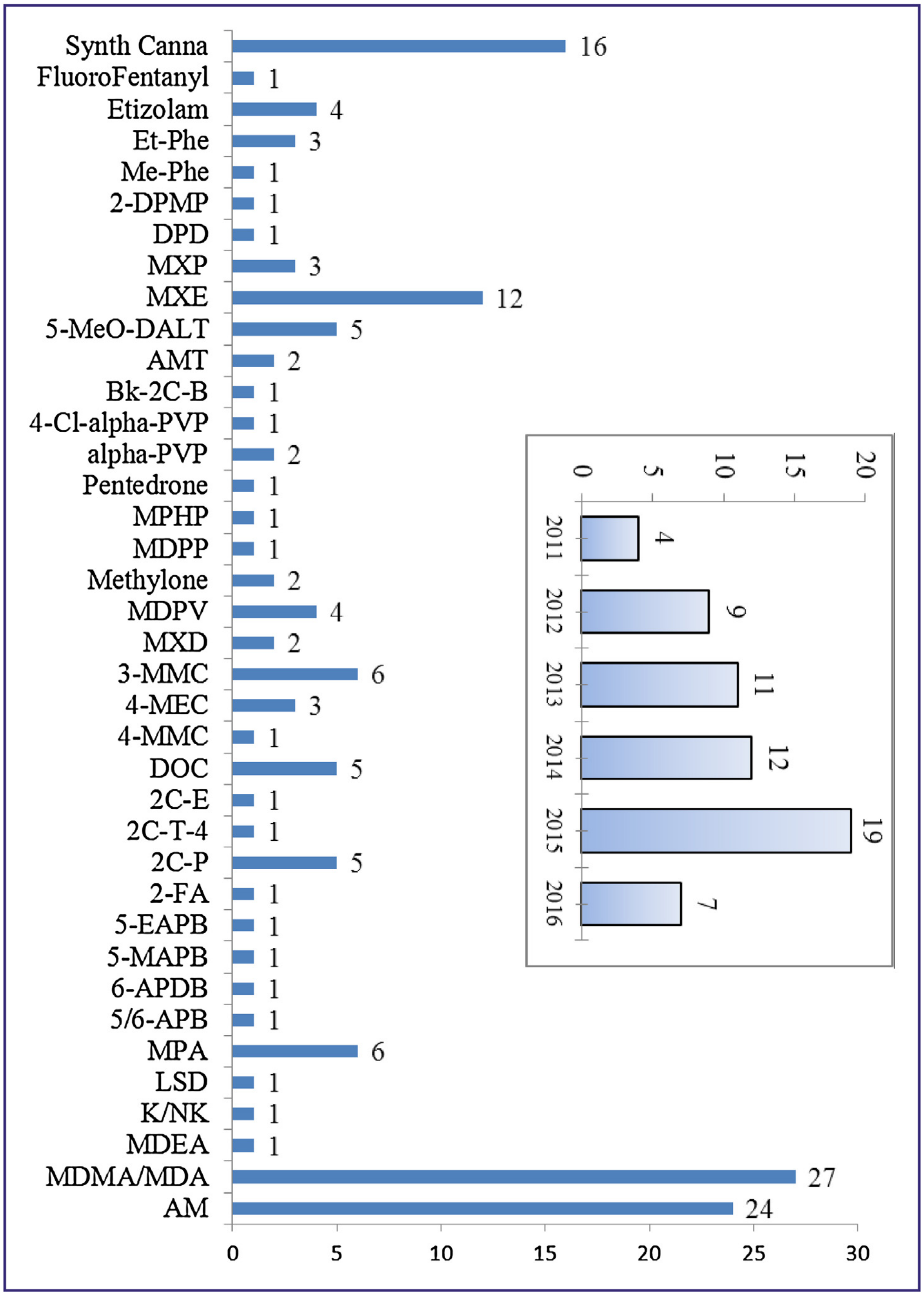

Figure 1. Frequency of new psychoactive substances and total annual distribution in cases observed between 2011 and 2016 compared with other illicit amphetamines or miscellaneous drugs over the same period.

(*n* = 17), respectively. Identification of some metabolites was possible by comparison with a phase I and II metabolism study using different CYP enzymes [28]. Methoxphenidine (MXP) was detected at concentrations of 254, 580 and 590 μg/L in plasma and of 4.1, 4.9 and 4.8 mg/L in urine and diphenidine (DPD) in only one case (plasma 20 μg/L) in combination with MPA and methadone. For this patient, the powder purity was higher than 95% expressed as chlorhydrate salt. Throughout this retrospective study, ketamine, not typically considered as NPS, was detected once (100 μg/L, norketamine 250 μg/L) in a case of DOC ingestion [25].

Miscelleanous compounds

Desoxypipradrol (2-DPMP) was observed in one case with a plasma level of 136 μg/L measured at D3 after ingestion and with a urine level of 4.8 mg/L on D4 [7,12]. Methylphenidate (MetPhe) was observed once with a 100 μg/L plasma level and its homolog ethylphenidate (EtPhe) three times (pl: 100, 160 and 1900 μg/L; ur: 7.2 and 120 mg/L). Etizolam, a benzodiazepine often described by users as complementary compound of the NPS consumption, was observed in 4 cases. Only one synthetic opioid was very recently detected in a powder as fluorofentanyl without determination of halogen

Table 2 Different synthetic cannabinoids detected over the 6-year review.

Chemical name	Code name	*n*
N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide	AB-Chminaca	1
N-(4-fluorobenzyl)-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(pentyl)-1H-indazole-3-carboxamide	AB-Fubinaca	1
N-adamantyl-1-(pentyl)-1H-indazole-3-carboxamide	AKB-48	1
N-adamantyl-1-(5-fluoropentyl)-1H-indazole-3-carboxamide	5F-AKB48	5
N-adamantyl-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide	5FUB-AKB-48	1
((1-methylpiperidin-2-yl)-methyl)-1H-indole-3-(1-naphthalenyl)-methanone	AM-1220	1
Methyl-2-(1-(cyclohexylmethyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate	MDMB-Chmica	2
Quinolin-8-yl-1-(5-fluoropentyl)-1H-indole-3-carboxylate	5F-PB22	3
1-(pentylindol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)-methanone	UR-144	1

position (meta- or para-). Due to the long delay to obtain the reference products, the biological samples of this case are still under investigation.

Synthetic cannabinoids (SC)

Over the 6 years, analysis of powders or brand liquid or plant samples available on the web market (Buddha blues, Laughin assassin, Pandora, MJSweet) obtained in 7 cases showed 9 different SC (Table 2) [19]. Their identification was possible basing on LC-UV retention times and spectra but many of them have similar UV profile. Their mass spectra after GC-MS were more specific and allowed accurate identification using the Cayman Chemical® library (free access on the web). All these compounds were confirmed by comparison with reference substances. However, it could be noted that the esters as 5F-PB22 were not well analysed by GC-MS because of thermal degradation or transesterification in methanol leading to 2 peaks. Even if the analysis of 15 different SC by LC-MS^2 in biological samples was not currently fully validated the estimated concentrations of the detected substances in our biological samples were presented in Table 1. In one case, a powder, contained in a plastic bag called ''Natrium 1 g or Ethylphenidate'', was found as a mixture of 5F-AKB48 (~ 43%), lidocaine and caffeine [19]. In another one, the self-made label on the plastic bag did not match with the detected compound as described below in selected case 2.

Some selected cases

Besides the already published cases, we have selected some typical cases.

Case 1

A 41-year old man was admitted to an ICU for agitation, hallucinations, tachycardia, hemodynamic instability and hypertension [16]. The Glasgow score (GS) was 11 and the patient was oliguric around 4 h after admission. The patient is suspected to ingest 20 mg of 2C-T-2 by oral route. The toxic effects disappeared slowly at H10 and the patient was discharged at H15. Urinalysis by LC-UV after alkaline and further acidic-back extraction showed fluoxetine and its metabolite (norfluoxetine) in therapeutic range, lidocaine (administrated for patient management at the care unit) and a peak at 7.5 min of which the observed spectrum matched perfectly with 2C-T-4 (Fig. 2). 2C-T2 had both a slightly but sufficiently different UV spectrum and a −0.7 min shift of retention time in the applied chromatographic conditions. Two other peaks at 3.26 and 3.9 min had similar spectra with 2C-T-4 suggesting possible metabolites. The identification of 2C-T-4 was unequivocally carried out using its GC-MS spectrum. This spectrum presented a +14 amu delta for the molecular HFB ion (m/z 451 vs 437) compared with HFB-2C-T-2 and the same shift for a fragment (m/z 225 vs 211). Analysis by LC-MS^2 allowed the quantification of 2C-T-4 (MRM transitions 256.1 > 197.1/256.1 > 239.2) at levels of 0.1 and 2.4 mg/L in plasma and urine, respectively.

Case 2

A 37-year old man known for drug abuse was admitted to an ICU [17]. Symptoms were somnolence, thoracic pain, palpitations and hypertension (155/109 mmHg). A possible ingestion of several NPS 1 and 7 hours before admission was suspected and 5 samples of powders (*n* = 4) or liquid (*n* = 1, some μL in a plastic bag) were obtained. The clinical symptoms disappeared quickly and the patient was discharged 1 day after. Initial qualitative cocaine and amphetamines immunochemical urine analyses were highly positive. Urinalysis by LC-UV/DAD showed many compounds with psychotropic drugs as cyamemazine, hydroxyzine and metabolites, citalopram and its N-demethyl metabolite but also levamisole in relation with consumption of cocaine, further confirmed by LC-MS^2 analysis. Four other compounds identified as MPA, responsible for amphetamine positive test, methoxetamine (MXE) and its desethyl metabolite, in addition to MXP (Figs. 3 and 4). Quantification of the 3 NPS in plasma and urine were respectively as following: 0.18 and 22.6 mg/L for MPA (MRM transitions 156.2 > 125/156.2 > 97), 0.12 and 13.8 mg/L for MXE and 0.25 and 4.9 mg/L for MXP (MRM transitions 296.2 > 211.2/296.2 > 129.1). Two powders, manually labelled ''MPA (1 g)'', or typographically labelled ''Methoxphenidine (not for human consumption, 1 g)'' were identified as highly pure substances if expressed as chlorhydrate salt. The third sample (labelled ''5F-UR144, XLR-11'') was identified as the pure non-fluorinated derivative, i.e. UR144 [19]. The fourth powder, ''labelled METHOX, 2 g, 4110PW2'' was identified as MXE at 90% purity. The liquid labelled ''NZT-48'' was a mixture of six compounds (MPA, caffeine, phenacetine, cocaine, 5-MeO-DALT and 5F-AKB48).

Case 3

A 26-year old man was admitted unconscious to an ICU with a GS 4/15, mydriasis, trismus and with seizures resistant

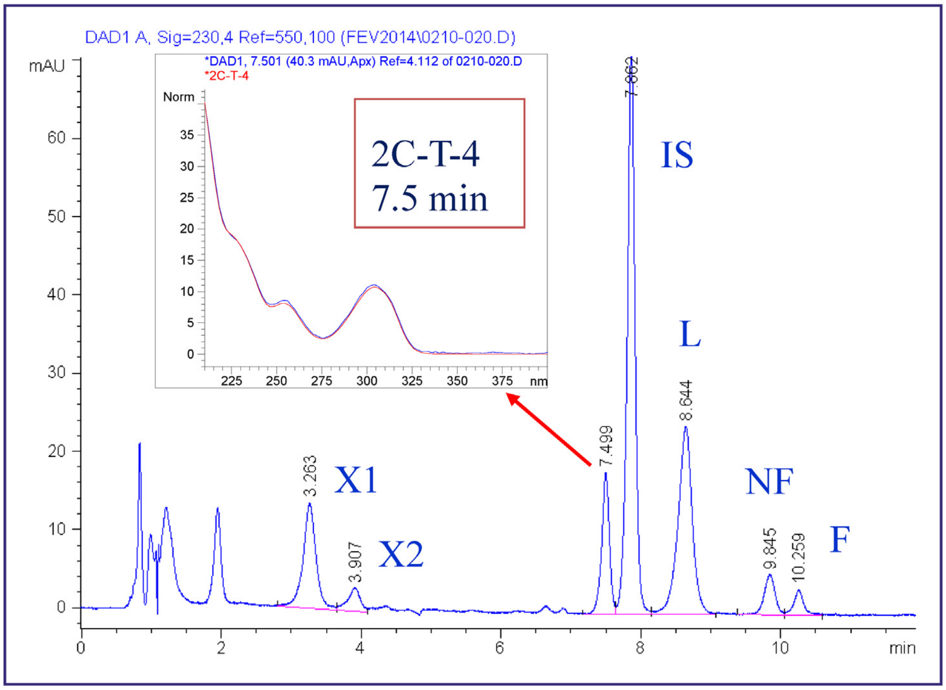

Figure 2. LC-UV chromatogram (230 nm wavelength) of 10-fold diluted urine sample of the ''selected case 1'' patient after alkaline extraction followed by acidic back-extraction [16]. IS: internal standard; L: lidocaine; F, NF: fluoxetine and norfluoxetine; X1, X2: supposed metabolites of 2C-T-4).

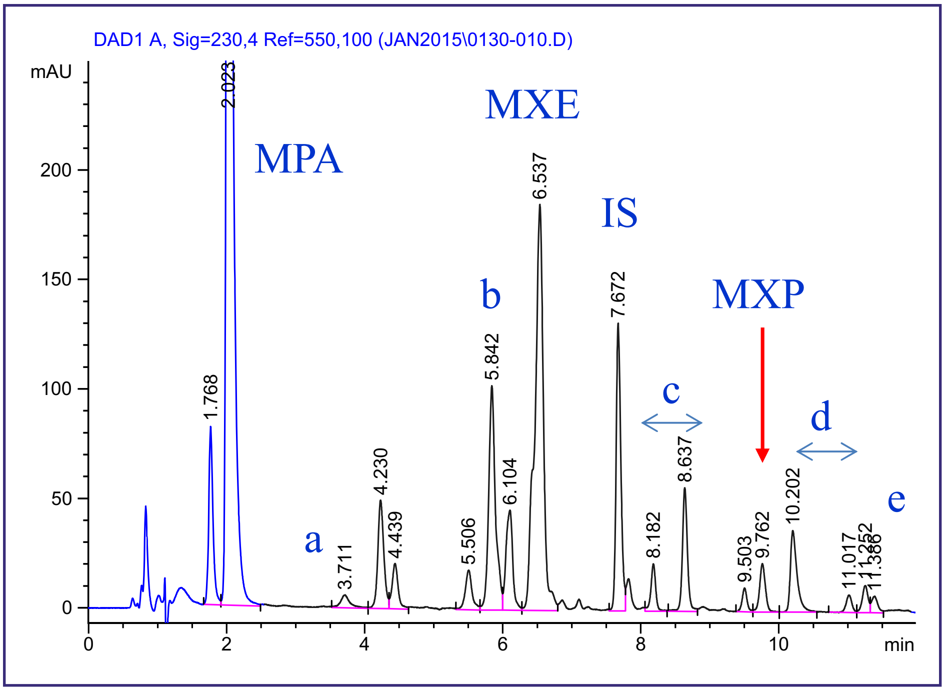

Figure 3. LC-UV chromatogram (230 nm wavelength) of five-fold diluted urine sample of the ''selected case 2'' patient after alkaline extraction followed by acidic back extraction [17]. MPA: methiopropamine; MXE: methoxetamine; MXP: methoxphenidine; IS: internal standard; a: levamisole; b: N-desethyl MXE; c: citalopram and metabolite; d: hydroxyzine and metabolites; e: cyamemazine.

to both clonazepam and diazepam therapy [16,17]. Consumption of alcohol and drugs of abuse was suspected. An ingestion of 5-MeO-DALT was suggested by his friends. At admission, blood alcohol was 2.2 g/L and amphetamine immunochemical urine analyses revealed positive reaction. The patient was extubated at H10 and discharged on day 2. Urinalysis showed MPA and 5-MeO-DALT confirmed both by their UV and MS spectra. The MPA-HFB derivative presented the 4 main characteristic ions with m/z 97, 124, 210, 154 where m/z 124 was the thiophene propyl fragment after

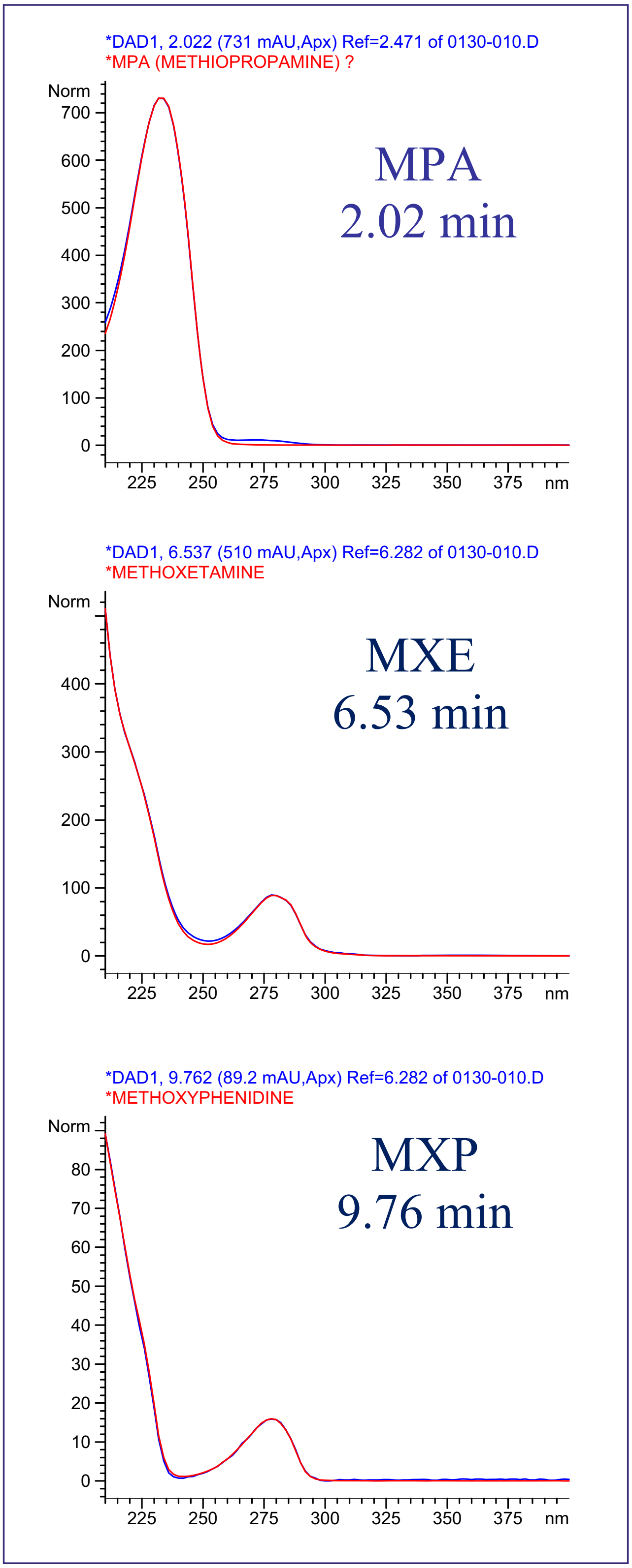

Figure 4. UV spectral comparison of 3 peaks from the ''selected case 2'' patient with reference methiopropamine (MPA), methoxetamine (MXE) and methoxphenidine (MXP) from the home-made library [17].

N-Cα breakdown, and m/z 97 was the thiophene methyl part after fragmentation between α and β carbon atoms and m/z 254 the nitrogen moiety with secondary fragmentation to m/z 210 as observed for metamphetamine. The 5-MeO-DALT non-derivatized spectrum presented the main m/z 110 moiety for the CH2-N-(CH2-CH = CH2)$_2$ fragment and the m/z 160 ion for the methoxy-indole part following fragmentation between Cα and Cβ bond. The m/z 270 parent ion is present but with a low intensity compared to the 100% ion. Plasma and urine 5-MeO-DALT concentrations were 0.03 and 1.6 mg/L (MRM transitions 271.2 > 110.1/271.2 > 174.2). MPA was found at the concentration of 0.05 and 13.4 mg/L in plasma and urine, respectively. The analysis of the powder showed a MPA and benzocaine mixture.

Case 4

A 38-year old man, known for drug abuse, was admitted to an ICU for agitation and hallucinations following consumption of a white powder bought on web market [21]. Symptoms were: hypersudation, bilateral mydriasis, tachycardia (106 b/min), hyperreflexia and tonic-clonic seizures. Initial biological analyses revealed a hypokaliemia (3.2 mM), elevated CPK (1593 UI/L) and hyperleucocytosis (12.6 G/L). Blood alcohol and urine drugs of abuse tests were negative. LC-UV analyses showed the same peak in urine and in the powder with a UV spectrum similar to pentedrone but with a different retention time. The identification as α-PVP was based on both analyses by GC/MS after HFBA derivatization (m/z 126 ion as observed for MDPV) and by LC/MS^2 (m/z 232 M + 1 ion [Delta -44 with MDPV]). This identification was further confirmed by comparison with the reference substance. Plasma and urine samples were analysed using a validated LC-2D-MS^2 method allowing the quantification of 5 PVP like-compounds and 3-MMC with MRM transitions as following: α-PVP: 232.2 > 90.9/232.2 > 126.3; Oxo-PVP: 246.2 > 160.9/246.2 > 91.1; 3-MMC: 178.2 > 160.1/178.2 > 145.0; α-PPP: 204.1 > 105/204.1 > 98.1; α-PVT: 238.2 > 126.3/238.2 > 167.1; 3,5-MeO-α-PVP: 292.2 > 151.1/292.2 > 221.1; IS: 261.1 > 244.1 (Fig. 5). α-PVP was not detected in plasma (LOQ 10 μg/L) and urine level was 11.9 mg/L while oxo-metabolite was measured in the 2 samples with levels of 57 and 568 μg/L. Another supposed metabolite was observed in LC-UV, corresponding probably to the hydroxy-metabolite.

Case 5

A 23-old woman, known for drug abuse, was admitted to an ICU for agitation and somnolence phases following a supposed ingestion of etizolam and possible 5-MAPB [21]. The GS was 10/15 with reactive myosis and the PSS was evaluated at 2. Blood ethanol was negative. The patient was discharged on the next day. 3-MMC was detected in plasma at 304 μg/L in addition to citalopram (20 μg/L), methadone (100 μg/L) and 5-MAPB (estimated concentration between 20—30 μg/L). No urine sample was available. It can be noted that 3-MMC and 4-MMC have the same chromatographic profile when analysed using LC-MS^2 with identical retention time and ion transitions upon the conditions described in case 4. However, these 2 meta- and para- isomers have significant different UV spectra that allow differentiation (Fig. 6).

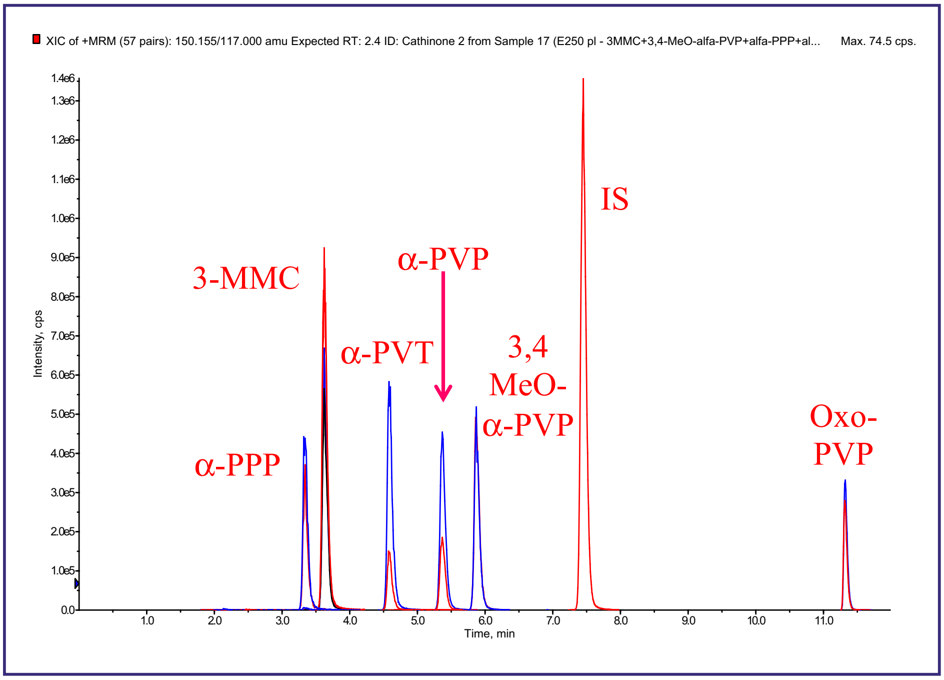

Figure 5. LC-2D-MS^2 of 3-MMC and PVP like-compounds (standards 250 µg/L plasma) on a Strata X precolumn coupled with a phenyl-hexyl elution column (analytical conditions described in text [21].

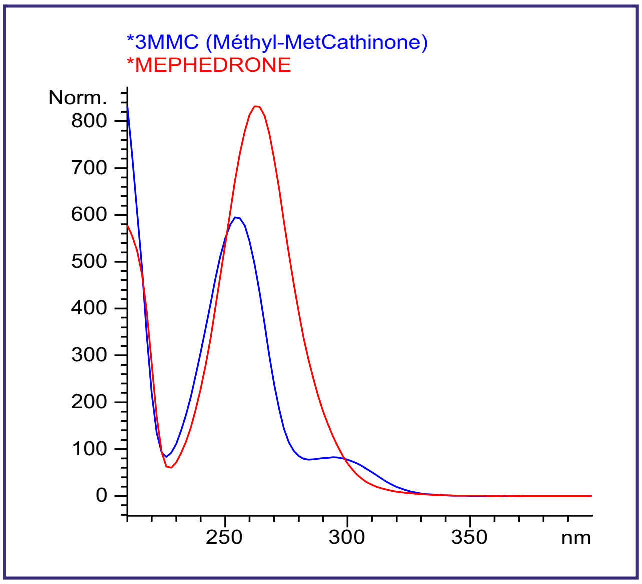

Figure 6. Differentiation of 3-MMC and mephedrone (4-MMC) using their UV spectra [21].

Discussion

This retrospective study collected data from a poison control center covering a large portion of the French territory and population (20%). Between 2011 and 2016, 41 different NPS were documented. For 3 of them, studies of their metabolism with identification of several metabolites were also performed using LC-high-resolution-MS^n [24,26,28]. The emergence of these new drugs is a real public health issue and it is very important to well document compounds and routes of consumption in order to establish therapeutic or preventive plans adapted to their consumers. This study highlighted the increase in consumption of these substances responsible for much intoxication which can be severe in young and healthy subjects. It is expressed by behavioural consequences (anxiety, psychosis, violence) and physical manifestations (hyperthermia, rhabdomyolysis, cardiovascular accidents, etc.). The management of patients is symptomatic. Our study showed that consumers had various experiences with NPS series and often with ingestion of several compounds belonging or not to the same family. Hence, the diagnosis of such intoxication is more difficult especially in poly-drug ingestion cases.

This review illustrated the challenge for clinicians and for analysts to provide well-documented information on these consumptions for a public health objective. In many hospitals, routine toxicological screening is generally not undertaken in such patients as the immediate clinical management of individual patients is not based on the results of toxicological analysis. Nevertheless, our study demonstrates that when toxicological screening is realized, with appropriate expertise, non-reported substances may be identified. Only few cases were associated with high toxicity and only one fatality was observed. These clinical data corroborated the numerous existing papers concerning recreational use or fatalities after NPS consumption [29—48]. Increasing documented cases about exposure to NPS should be the first step for a possible scheduling of these products as illegal drugs by Agence nationale de sécurité des médicaments (ANSM), the French Health authority agency.

Close collaboration between clinicians, physicians and analytical toxicologists is essential to better manage NPS consumption cases. Intoxications most commonly affect young subjects who can easily obtain compounds online or through widespread dissemination at both public and private parties. For consumers, knowledge of the danger associated with the use of these substances is limited and often trivialized. The results of this study should contribute to the implementation of a proactive policy against drug abuse.

Disclosure of interest

The authors declare that they have no competing interest.

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ORIGINAL ARTICLE

Poisoning by synthetic cathinones: Consumption behaviour and clinical description from 11 cases recorded by the Addictovigilance Centre of Bordeaux

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Received 14 October 2016; received in revised form 24 November 2016; accepted 25 November 2016
Available online 27 January 2017

KEYWORDS
Cathinone;
Poisoning;

Summary Synthetic cathinones represent a large class of new popular drugs of abuse in France and in Europe, commonly called new psychoactive substances (NPS). We describe 11 cases of

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http://dx.doi.org/10.1016/j.toxac.2016.11.005
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Substance-related disorders;
Public health surveillance

synthetic cathinone abuse reported to the Bordeaux Addictovigilance Centre between January 2014 and December 2015. Cases were described according to the type of substance used, patient's age and gender, type of complications, context of use and year of the event, and, when a toxicological analyse was available, the analytical method of detection and the samples of analyse (blood, urine, powder). All patients were male, with a mean age of 35.9 years old. The synthetic cathinone most frequently reported by users was methylenedioxypyrovalerone ($n = 8$). There was analytical confirmation of synthetic cathinone use in 6 cases. Nine cases were serious, with neurologic and/or psychiatric complications in most cases. The outcome was favourable in 10 cases but one patient died. Questioning patients about use of NPS is essential, as well as NPS analytical identification when an intake is suspected.

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Introduction

In recent decades, the psychoactive substances market has changed. Cannabis, opiates and cocaine are still the most commonly used psychoactive compounds in France (excluding tobacco and alcohol), but the use of MDMA (ecstasy) [1] and new substances are dramatically increasing, especially among young people in recreative places. These new substances, so-called new psychoactive substances (NPS) or research chemicals (RC), have appeared over the past 10 years, mainly because of reduced availability or quality of more ''classical'' substances such as heroin, cocaine or ecstasy, and the development of forums for users and shopping sites on the Internet. These NPS are chemical analogs or derivatives of substances already known, by adding a chemical radical to create several new derivatives with similar psychoactive properties. Because the chemical structure is different from the original substance, these new substances are not subjected to the regulations by law as it is the case for the original substance usually classified as narcotic. This feature is probably contributing to their availability on the market and increasing their consumption [2].

There are over a hundred NPS already known, consumed for their stimulant and entactogenic properties [2]. The prevalence of the use of these NPS is difficult to estimate. In the United Kingdom, a study among students highlighted that 20% of them had used at least once mephedrone [3]. A European study showed that 9% of emergency department income involving a psychoactive substance was related to the use of an NPS, and mainly cathinones [4]. In France, this was encountered for 7.4% of emergency department income involving psychoactive substance [5]. Synthetic cathinones, derived from khat [6], are the most frequently consumed NPS in France and Europe [4]. The increase in their use, especially among young people, led the French authorities to enroll some of these NPS on the list of narcotic substances in July 2012, as it was done before for cathinone and khat [6].

It is difficult to estimate the prevalence of cathinone complications mainly because patients at the emergency department are rarely questioned about the use of these drugs, and the NPS are not always detectable in biological samples by conventional toxicological screening usually used in laboratories [gas chromatography-mass spectrometry (GC-MS), liquid chromatography-diode-array detector (LC-DAD) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)]. For these reasons, the consumption is rarely known, and the clinical signs are rarely described.

In the present study, we report a series of 11 cases of synthetic cathinone consumption initially recorded by the Bordeaux Addictovigilance Centre [7], between January 2014 and December 2015.

Method

The French Addictovigilance network (FAN), coordinated by the French Medicines Agency (ANSM), monitors addiction related to psychoactive drugs (tobacco and alcohol excluded). The surveillance system, in place since 1990, is based on spontaneous reporting (mandatory for health professionals) and on specific surveillance programs using several data sources to monitor abuse potential of psychoactive drugs [7]. This participates to the risk assessment of the use of psychoactive drugs, of drug abuse and of consumption patterns for general public health.

For this study, all cases of synthetic cathinones consumption recorded by the Bordeaux Addictovigilance Centre were analysed between January 2014 and December 2015. Cases were described according to the type of substance used, patient's age and gender, type of complications, context of use and year of the event, and, when a toxicological analyse was available, the analytical method of detection and the samples of analyse (blood, urine, powder).

Results

In this 24-month period, 11 cases of poisoning with a synthetic cathinone were reported: 4 in 2014 (that represented 3.2% of all cases recorded by the Addictovigilance

Table 1 Cathinone cases.

Number	Sex, age (Y)	Type of Cathinone (administration route)	Medical history, comorbidities	Co-exposure	Clinical signs	Clinical outcome	Analytical method	Socio-economical context	Comments
Cases with toxicological analysis									
1-2014	M, 28	MDPV (IV)	HIV, HCV infection	Buprenorphine	Hallucinations	Recovery	HPLC/DAD, HPLC-MS/MS (syringe)	Squat, no professional activity	Intentional use of cocaine, large amount (low price), notion of occurrence of hallucinations in other residents of the squat, same context as 2-2014
2-2014	M, 35	MDPV (IV)	HIV, HCV, HBV infection	Buprenorphine	Stroke (middle cerebral artery)	Recovery	HPLC/DAD, HPLC-MS/MS (syringe)	Squat, no professional activity	Same context as 1-2014
3-2014	M, 47	Mephedrone (Und.)	Bone graft	Methoxetamine, cannabis	Vigilance disorders, Glasgow score 12/15, nystagmus	Recovery	HPLC/DAD, HPLC-MS/MS (plasma, urine)	Stable accommodation, professional activity, lives in couple	Und.
4-2015	M, 55	4-MEC (IV)	HIV infection	None	Abdominal pains, diarrhoea, hypotension, sudden cardiac arrest	Death	13 ng/mL HPLC/DAD, HPLC-MS/MS (plasma)	Professional activity	Injections during several days
5-2015	M, 30	MDPV (Und.)	Und.	GHB	Hallucinations, paranoia, tachycardia (112/min), hypertension (16/10), amnesia	Recovery	97 ng/mL LC-MS/MS (blood)	Und.	Und.

Table 1 (*Continued*)

Number	Sex, age (Y)	Type of Cathinone (administration route)	Medical history, comorbidities	Co-exposure	Clinical signs	Clinical outcome	Analytical method	Socio-economical context	Comments
6-2015	M, 47	bk-2C-B (oral)	HIV infection	None	Dizziness with loss of consciousness, empty gaze, stereotyped movement of the trunk, dystonia muscularum, reactive bilateral mydriasis	Recovery	LC-DAD (plasma, urine, powder)	Und.	Purchase on the Internet
Cases without toxicological analysis									
7-2014	M, 44	MDPV (nasal and oral)	Und.	None	Psychomotor agitation, insomnia, craving, paranoia, anorexia	Recovery	No	Stable accommodation, professional activity, lives with his children	Purchase on internet
8-2015	M, 20	MPDV (Und.)	Und.	None	Logorrhoea, tachycardia (120/min), hypertension (126-87)	Recovery	No	Stable accommodation, student, lives in couple	Hazardous sexual behaviour
9-2015	M, 25	MDPV, 3-MMC, 4-MEC (nasal)	HIV infection	None	Hallucinations with the sensation of peeling off the skin, paresthesia	Recovery	No	Stable accommodation, lives alone	Und.

Table 1 (*Continued*)

Number	Sex, age (Y)	Type of Cathinone (administration route)	Medical history, comorbidities	Co-exposure	Clinical signs	Clinical outcome	Analytical method	Socio-economical context	Comments
10-2015	M, 30	MDPV (IV, oral)	HIV, HCV infection, Crohn's disease	None	Psychomotor agitation, visual and somesthetic hallucinations, anorexia, insomnia	Recovery	No	Stable accommodation, lives alone, unemployment	Purchase from person buying on the internet (bath salts), 1 g during 6 days, several times a day
11-2015	M, 29	MDPV, Mephedrone (IV, nasal)	HIV infection, syphilis, bicuspid aortic valve, infectious endocarditis	None	Recurrent infectious endocarditis, drug addiction	Recovery	No	Stable accommodation, professional activity, lives alone	Slam

4-MEC: 4-methyletcathinone; bk-2C-B: 2-amino-1(4-bromo-2,5-dimethoxyphenyl)ethan-1-one; GHB: γ-hydroxybutyric acid; IV: intravenous injection; M: male; MDPV: methylenedioxypyrovalerone; HBV: hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; min: minute; Und.: undetermined.

Centre, $n = 124$) and 7 in 2015 (that represent 3.7% of all cases recorded by the Addictovigilance Centre, $n = 188$) (Table 1). Seven patients have been admitted in an emergency service, one in an intensive care unit, one in a psychiatric department, two in a department of internal medicine. Among cases, 9 were serious, according to the international classification [8]. All consumers were men with an average of 35.9 years old (median: 35 years; range, 20—55 years). Two patients were homeless without professional activity. In two cases, synthetic cathinone was consumed in a context of sexual behavior which one was related to slam (intravenous psychoactive substance injection during sexual party especially among men who have sex with men [9]). Seven patients were HIV positive, and a co-infection with hepatitis C (HCV) was reported in 3 cases. Serology for the hepatitis B virus (HBV) was positive in 1 case. The synthetic cathinones declared to be consumed were methylenedioxypyrovalerone (MDPV) in 8 cases, mephedrone in 2 cases, 4-methyletcathinone (4-MEC) in 2 cases, 3-methylmethcathinone (3-MMC) in one case, 2-amino-1(4-bromo-2,5-dimethoxyphenyl)ethan-1-one (bk-2C-B) in one case. In 2 cases, patients had consumed several synthetic cathinones in once. In 6 cases, toxicological analysis confirmed the presence of a synthetic cathinone. In other cases, patients had reported taking a synthetic cathinone, but unfortunately, there was no analytical confirmation. In two patients who thought they had taken cocaine, MDPV instead of cocaine was found. The synthetic cathinones were consumed by intravenous injection (IV) in 5 cases, by nasal route in 3 cases, and by oral route in 3 cases. For 3 cases, the route of administration was not specified, whereas multiple routes were used in 3 cases. In 4 cases, synthetic cathinones were associated with buprenorphine, γ-hydroxybutyric acid (GHB) and methoxetamine. Patients were admitted to emergency department for neuro-psychiatric disorders in 10 cases, and for recurrent infectious endocarditis in one case. A summary of the different cases is presented in Table 1. Clinical outcome was favorable for 10 patients, but unfortunately, one died of a severe hypotension and cardiac arrest.

Discussion

In this study, we reported 11 patients who were hospitalised in emergency department after known or suspected synthetic cathinones use. Compared to 2014, the number of recorded cases to the Bordeaux Addictovigilance Centre has been doubled in 2015, which may be related to an increase in consumption. Nevertheless, among all cases of psychoactive substances use recorded at the Bordeaux Addictovigilance Centre, the proportion of NPS remained comparable between 2014 and 2015. The consumption of these psychoactive substances is often subject of questions to the Addictovigilance Centres or to the Poison Control Centres from physicians who are less familiar with these new substances as compared to more conventional ones such as cocaine or heroin. In general, in Europe, this consumption is steadily increasing [4]. Since 2004, when were identified the first synthetic cathinones, 103 new synthetic cathinones were identified, including 26 in 2015 [4]. The prevalence of the consumption in the general population is very difficult to assess, especially since the consumer profiles are extremely different, from the homeless patient to the well socially integrated people.

All of our patients were men, but their way to use NPS, even if they were not always detailed, seemed quite different. The first two cases differ slightly from the others as they were taking MDPV unintentionally, unlike the other cases. Indeed, those patients, homeless, thought they had bought cocaine. As they experienced some hallucinations, as others consumers form their squat, a toxicological analysis was performed on the remaining liquid in the syringes, which confirmed the absence of cocaine but the presence of MDPV. Thus, the identification of the psychoactive substances in biological samples (blood, urine) is essential to ascertain if the consumed substances are really the substances that the patients think to have consumed but also to impute the symptoms presented by patient to the real consumed substances. Indeed, it is noteworthy that for any product purchased on the Internet, the composition is not guaranteed. Identification in biological samples could also help to highlight which NPS are on the marketplace.

The route of administration was extremely variable and appeared to depend on the patient.

Common modalities of intake are through snorting intranasal (insufflation) or by oral ingestion by either swallowing capsules, ''parachuting'' (wrapping powder in cigarette papers and swallowing) [10] or dissolving it in liquid [11]. Less common methods may include rectal administration and intravenous use. In a recent study, the use of cathinones [12] was described in 14 patients supported in a drug dependence care centre. All were homosexual men, consuming the products predominantly by intravenous injection to experience SLAM [9]. Psychoactive substances used for SLAM are mostly synthetic cathinones. Other substances or drugs may be associated like GHB, γ-butyrolactone (GBL, a GHB precursor), poppers, cannabis, phosphodiesterase inhibitors, etc. In one of our cases, the cathinone was used in a slam context, and another one was suspected for slam but we did not confirm this sexual use due to missing data. In 3 cases, cathinones were purchased on the Internet, which constitute an easy access to the psychoactive substances especially for those who are not used to buy those substances in the street.

Like amphetamines or cocaine, synthetic cathinones act as stimulants for the central nervous system, increasing synaptic concentrations of catecholamines (dopamine, serotonin, norepinephrine). They inhibit the catecholamine reuptake, but also stimulate the release of those monoamines [6]. In animals, synthetic cathinones increase the locomotor activity, inhibit food intake, and are involved in motor coordination and thermoregulation [6]. In humans, synthetic cathinones have stimulant and entactogenic properties [6]. Effects include euphoria, increased sensations, mental stimulation, increased sociability, empathy, and sexual excitation. Clinical effects are reported to sympathomimetic and serotonergic ones and are responsible for many clinical signs including cardiovascular (tachycardia, arrhythmias, hypertension), neurological (agitation, headache, confusion, convulsions) and psychiatric ones (hallucinations, anxiety with suicidal thoughts, insomnia, violent behaviour disorders, bruxism). General signs are also observed like anorexia, malignant hyperthermia, and rhabdomyolysis. Cardiovascular collapses and convulsive comas were also described with cerebral edema, multiple organ failure and death [6]. This is coherent with the effects observed in the present study for the patients for whom a toxicological analysis has been performed (hallucinations, stroke, vigilance disorders, cardiac arrest, tachycardia, other neurologic disorders). For the others, the effects were compatible with the intake of a stimulant, cathinones included, as symptoms were not specific.

Clinical outcomes varied among patients, most probably because of inter-individual variation: the first two cases were of particular interest because both patients had taken the same amount of psychoactive substance, in the same conditions but were not hospitalized for the same reasons. In addition, it is likely that clinical effects may vary over the period of time, or according to the nature of the cathinone, the dose or the possible association of several of them, or even the combination with other psychoactive substances from another class. If all cathinones may increase locomotor activity, they do not have the same efficiency, amplitude of stimulation, or duration of action [13]. Warm ambient temperature may also have a role in the stimulation of motor activity following administration of synthetic cathinones [13]. In the present study, it was unfortunately impossible to make correlations between the amounts of substance taken, the plasmatic concentrations and the observed effects, because only drug identifications by LC-MS/MS were performed. Moreover, information about time to onset of the effects, pattern of consumption are often missing in the discharge summary consulted retrospectively when assembling the addictovigilance file.

There is no specific antidote to cathinones intoxication and the treatment is symptomatic. As observed in the present study, the risk of infectious diseases is important, because of the risk of HIV or hepatitis C virus transmission by contaminated needles exchange or during slam with sexual trauma. An infectious risk also exists when using intravenous injection without disinfection of needles (abscess, septicemia, endocarditis, etc.). In the present study, one case of recurrent infectious endocarditis was observed, probably more due to the route of administration than to the substances themselves (mephedrone or MDPV). Cases of addiction were also described in the literature [14], as it was the case for 2 of the patients in this series, and a craving is particularly related with the use of these substances.

Conclusion

Increase in hospitalizations after synthetic cathinone consumption is particularly worrying, and shows the ineffectiveness of the systematic prohibition of these substances. When a patient is suspected to have consumed NPS, the questioning is essential. These new substances must not be forgotten by the physicians, especially when analysis is prescribed. Biological sampling (urine and blood) is essential for the analytical evidence of poisoning by the NPS in order to improve the clinical description of these poisonings, their management and to describe the substances in the French drug market. Indeed, the clinical signs, including psychiatric disorders, can be difficult to manage in an emergency department and could create some difficulties for emergency medical team.

Moreover, it seems likely that there is an underestimation of cases. Education of physicians from emergency departments, in addition to professional working in drug dependence care centres or harm reduction centres and associations such as AIDS that are already highly mobilized on the slam is primordial. In addition, to our point of view any hospitalization associated with the consumption of these NPS must be reported to improve the knowledge about the nature of these psychoactive products and their toxicity, as well as to assess the extent of their consumption.

Disclosure of interest

The authors declare that they have no competing interest.

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Toxicologie Analytique & Clinique (2017) **29**, 41—46

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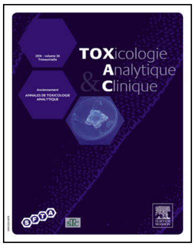

ORIGINAL ARTICLE

New psychoactive substances in a drugged driving population: Preliminary results

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Received 7 November 2016; received in revised form 9 December 2016; accepted 10 December 2016
Available online 29 December 2016

KEYWORDS
Driving under the influence of drugs;
New psychoactive substances;
LC-MS/MS;
LC-HRMS

Summary Action against driving under the influence of drugs (DUID) often starts with an on-site immunological screening which is not yet developed for new psychoactive substances (NPS). Our aim was to determine the prevalence of NPS in drivers screened positive for a classical illicit drug. Blood samples ($n = 556$) were obtained between January—August 2015 in Belgium. The on-site Drugwipe 5S (Securetec) results and the subjects' signs of recent drug use were available. Classical illicit drugs were confirmed in blood via LC-MS/MS methods. NPS screening was performed using 2 methods: LC-HRMS and LC-MS/MS. Of the 256 samples yet analysed, NPS were detected in 13 samples (5%) including following substances (n): ketamine (5), methoxetamine (2), diphenidine (2), 5-MeO-DALT (1), 4-AcO-DiPT (1), methiopropamine (1), methedrone (1), α-PVP (1), a mix of 5-MAPB/5-EAPB (1), and AB FUBINACA (1). This preliminary study demonstrates a prevalence of 5%. However, this result should be confirmed and reassessed at the end of our study.

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http://dx.doi.org/10.1016/j.toxac.2016.12.002
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Introduction

A challenging issue for drafting international and European drug policies is how to respond effectively to the increasing and dynamic new psychoactive substances (NPS) market. NPS, including synthetic cannabinoids, synthetic cathinones, phenethylamines, opioids, tryptamines, benzodiazepines and arylalkylamines are mainly sold in Internet Shops. For instance, 98 new substances were detected for the first time in the EU in 2015, bringing the number of new substances monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) to more than 560, of which 380 (70%) were detected in the last 5 years [1,2]. In this situation, one main challenge consists in the identification of NPS in biological samples (blood, urine, hair, oral fluid) in clinical and forensic cases, e.g., intoxications or driving under the influence of drugs (DUID).

Action against DUID often starts with an on-site immunological screening focusing on classical drug of abuse (DOA): i.e. cannabis, cocaine, amphetamine or heroin use. These rapid tests are not yet developed for NPS. Actually, in Belgium as well in France, roadside testing is performed in oral fluid using an on-site immunological screening for DOA: Drugwipe® 5S — Securetec. In the case of a positive result, a blood sample is subsequently collected for confirmation using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) [3].

The aim of this study is to determine the prevalence of NPS in drivers screened positive for classical illicit drugs.

Material and methods

Plasma samples were obtained between January and August 2015 in Belgium in 3 geographic areas (Bergen [Mons], Mechelen [Malines] and Turnhout [Turnhout]) mainly following unexpected roadside controls.

The on-site Drugwipe 5S (Securetec) results and the subjects' signs of impairment were available. After a dual (Brussels and Lille) confirmation step for usual drugs of abuse using liquid chromatography and tandem mass spectrometry detection (LC-MS/MS) in Multi Reaction Monitoring (MRM) mode methods [4—7], screening for NPS was performed using 2 previously reported methods [8,9]: (M1) liquid chromatography and high resolution mass spectrometry detection (LC-HRMS) with a Xevo-G2 QTOF-XS system (Waters, Manchester, UK), and (M2) an LC-MS/MS using a XEVO TQS system (Waters, Manchester, UK).

Briefly, for M1, 400 μL of sulfosalicylic acid (3%) was added to 100 μL of blood. After on-line extraction using OASIS® HLB (Waters), separation was performed on a Acquity™ UPLC HSS C_{18} column (Waters) and detection occurred in MS^e (ESI+) mode. Data processing was performed using ChromaLynx™, TargetLynx™, MassFragment™ and MetaboLynx™ associated softwares (Waters) using a homemade database of more than 1.400 substances including nearly 300 NPS or metabolites. For M2, 100 μL of blood sample was deproteinated (300 μL methanol), diluted (100 μL of ammonium formate/0.1% formic acid buffer), and analysed after separation by an Acquity™ UPLC BEH C_{18} column (Waters). Detection occurred in the MRM mode (ESI+) for 222 NPS or metabolites.

Results and discussion

Between January and August 2015, 556 collected blood samples were taken after positive roadside tests. To date, 256 have been analyzed. These 256 samples represent about 8% of all collected blood samples in Belgium after positive roadside testing over the period considered.

These samples were obtained from DUID controls (98%) or after minor accidents (2%). The Drugwipe 5S tests in oral fluid related to these samples were positive for THC (76%), cocaine (19%), amphetamine (16%) or MDMA (8%) either for one parameter or in combination.

NPS were detected in 13 of 256 samples (5%) including following substances (*n*): ketamine (5), methoxetamine (2), diphenidine (2), 5-MeO-DALT (1), 4-AcO-DiPT (1), methiopropamine (1), methedrone (1), α-PVP (1), a mix of 5-MAPB/5-EAPB (1), and AB FUBINACA (1). The results of theses 13 cases are presented in Table 1. Noteworthy are the facts that (i) all users are male and this is in coherence with previously published data reporting that the vast majority of 'legal-high' users were men [10], (ii) 6 of these 13 NPS positive cases (46% of positive cases) were obtained from a control nearby a festival site (the music festival of DOUR), and (iii) these 13 drivers admitted their classical drug use, detected with the on-site screening, but they did not mention their NPS use. This latter fact leads us to the question whether drug users always know what they are using or if it is due to a direct confrontation with a test result that they are willing to confirm there drug use.

Among the results, we want to focus on case #4 in which 5-MeO-DALT was detected in blood after a false-positive cocaine on-site screening in oral fluid (Fig. 1). There are no data in the literature about false positive result of cocaine immunoassay-based tests due to 5-MeO-DALT and, more broadly, tryptamine derivatives seem not detectable with common applied immunoassay-based techniques [11]. In order to investigate this case, we had performed an assay with a blank oral fluid specimen spiked with 5-MeO-DALT (1 g/L) using an *ad hoc* testing device (Drugwipe® 5S — Securetec) at our disposal: the obtained result was positive for cocaine. So, the possibility of positive result of an immunoassay-based test for cocaine due to the presence of 5-MeO-DALT in oral fluid should be considered, but requires further studies to be confirmed.

Lastly, in the challenging NPS area with an increasing and dynamic market, it is not possible to be sure that no NPS was missed by the used analytical techniques, even if the used LC-HRMS method exhibits acceptable analytical criteria for drug sample identification regarding to literature recommendations in this context [12,13].

It is difficult to assess the meaning and impact of the observed NPS prevalence (5%) in this drugged driving population. Indeed, it is challenging to compare these preliminary results to data found in the literature due to the differences in studied populations and applied analytical tools [14]. Nevertheless, the following data can be considered. In the EU, the NPS prevalence is estimated by the 2014 Flash Eurobarometer, a survey of over 13 000 young adults aged 15—24 in the EU Member States, which asked about their NPS use. Eight percent of respondents had used an NPS at least once, with 3% in the last year. The countries with the highest levels of use in the last year were in Ireland (9%),

Table 1 NPS positive cases of drivers screened positive for classical illicit drugs.

#	LA	Case information	Blood results		
			DOA	NPS	Others
1	TU	Drug control/Drugwipe positive for cocaine Drug use admitted: cocaine Medical information: hypercholesterolemia	Cocaine, BZE, EME, cocaethylene amphetamine	Ketamine (and norketamine)	Levamisole Tetrazepam Quetiapine (and metabolite)
2	TU	Drug control/Drugwipe positive for cannabis Drug use admitted: daily use of cannabis.	THC, 11OH THC, THCCOOH	MXE (and a desmethyl metabolite) diphenidine (and dihydroxy metabolite)	None
3	ME	Drug control/Drugwipe positive for cannabis and amphetamine Drug use admitted: use of speed Checklist: mydriasis, dry mouth/lips, resignation, admits drug use Medical information: use of bromazepam in the past	THCCOOH Amphetamine	MXE	None
4	ME	Drug control/Drugwipe positive for cocaine Drug use admitted: none Checklist: shiny eyes, mydriasis, pale skin tone, resignation, euphoria, repetition of words, talkative Medical information: very aggressive and impolite behaviour	None	5-MeO-DALT (and a hydroxydesalkyl metabolite)	None
5	MO*	Drug control/Drugwipe positive for cannabis and amphetamine Drug use admitted: 1 joint 1–6 h before control, and speed 1–6 h before control Checklist: shiny eyes, dry mouth/lips, agitation, walks too sure of himself, admits drug use negative for alcohol, claims not to have taken medication, claims to come from the DOUR festival	THC, 11OH THC, THCCOOH Amphetamine	Ketamine (and norketamine)	None
6	MO*	Drug control/Drugwipe positive for cocaine and amphetamines Drug use admitted: 1 XTC tablet 1–6 hours before the control, 1 g cocaine 1–6 h before the control Checklist: shiny eyes, myosis, dry mouth/lips, pale skin tone, agitation, talkative, nervous tics, admits drug use, drug odour, walks too sure of himself, declares no use of medication, negative for alcohol, claims to come from the DOUR festival	Cocaine, BZE, cocaethylene MDMA, MDA	5-MAPB 5-EAPB	None
7	MO*	Drug control/Drugwipe positive for cannabis Drug use admitted: 2 joints marijuana 6–12 h before the control Checklist: shiny eyes, pale skin tone, fatigue, admits drug use, drug odour, drug possession, declares no use of medication, negative for alcohol, claims to come from the DOUR festival	THC, 11OH THC, THCCOOH Cocaine, BZE	Ketamine (and norketamine)	Quinine quinidine

Table 1 (*Continued*)

#	LA	Case information	Blood results		
			DOA	NPS	Others
8	MO*	Drug control/Drugwipe positive for cannabis, cocaine, amphetamines Checklist: myosis, pale skin tone, agitation, mental confusion, exaggerated reflexes negative for alcohol, DOUR festival	THC, 11OH THC, THCCOOH cocaine, BZE amphetamine, MDMA, MDA	Ketamine (and norketamine)	Alprazolam
9	MO*	Drug control/Drugwipe positive for cannabis Drug use admitted: ½ joint 1—6 h before control Checklist: shiny eyes, pale skin tone, tired, admits drug use, drug odour, drug possession, negative for alcohol, claims to come from the DOUR festival	THC, THCCOOH	MPA AB FUBINACA(and hydroxy + hydroxydesamino metabolites)	lidocaine
10	MO*	Drug control/Drugwipe positive for cannabis Drug use admitted: 2 joints the day before and 1 joint in the morning Checklist: resignation, admits drug use, drug odour negative for alcohol Medical information: Sertraline 100 mg (1/day), Remergon 30 mg (1/day), Theralene 0.5 mg/day and Xanax 1 mg (1/day); hepatitis B	THC, 11OHTHC, THCCOOH Cocaine, BZE	Methedrone	Alprazolam sertraline
11	TU	Accident (car damage, no physical damage)/Drug control/Drugwipe positive for cannabis Drug use admitted: 2 joints 2 days ago Medical information: Pantomed, NSAID	THC, 11OHTHC, THCCOOH	Diphenidine (and hydroxy + dihydroxy metabolites)	Tramadol (and metabolites) paracetamol
12	MO	Drug control/Drugwipe positive for cannabis Drug use admitted: 24/01/15 (18—19 h) 1 joint > 12 hours before control Checklist: shiny eyes, pale skin tone, fatigue, admits drug use, drug odour, drug possession, negative for alcohol, declares no medication use	THC, 11OHTHC, THCCOOH	Ketamine (and norketamine)	None
13	MO	Drug control/Drugwipe positive for cannabis Drug use admitted: 2-3 times a month a joint + 1 joint 1—6 h before control Checklist: mydriasis, dry mouth/lips, apathy, changing emotions/mood, constantly repeating his words, admits drug use, drug odour, drug possession, declares no medication use, visited a friend.	THC, 11OHTHC, THCCOOH	Alpha-PVP	None

LA: localisation area of sampling; ME: Mechelen (Malines); MO: Bergen (Mons); MO*: Bergen (Mons), close to the music festival of Dour; TU: Turnhout.

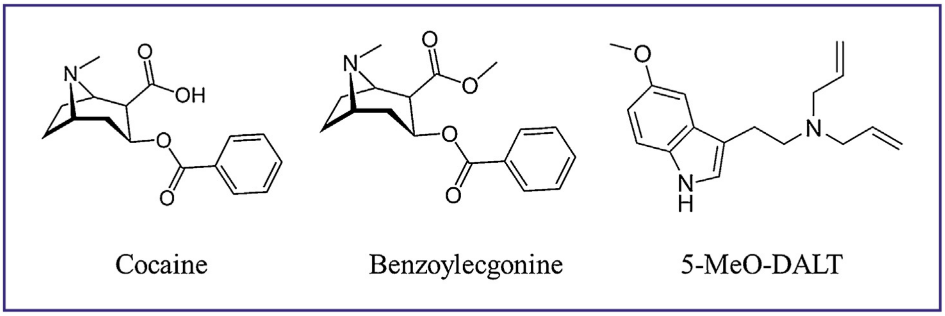

Figure 1. Structure of cocaine, benzoylecgonine and 5-MeO-DALT.

Spain and France (both 8%), and Slovenia (7%); Belgium was marked on the 12th place with 3%, while Malta and Cyprus concluded that no NPS were used by young adults [15]. At the same time, according to the Vereniging voor Alcohol- en Andere Drugproblemen (VAD; Association for Alcohol and other Drug-related problems), 0.1% of the Belgium population aged 15—64, and 0.3% of the Belgium population aged 25—34, had used NPS in the 12 last months in 2013 [16].

There are some reports of positive case series of impaired drivers (i.e. for synthetic cannabinoids), but without any prevalence value [13,17—19]. Only a few reports concerned NPS prevalence in the general drugged driving population. For instance, in Norway, for two periods of three/four weeks, all blood samples from drivers suspected of DUID were analyzed for the presence of synthetic cannabinoids. A total of 726 cases were collected and analyzed, and synthetic cannabinoids were detected in 16 cases (2.2%) [20]. In the same state, the same authors reported methiopropamine presence at frequency of 0.8% in DUID positive cases during a 12-week period [21]. Furthermore, during the three-year period from 2012 to 2014, in Poland 7000 cases were analyzed, resulting in 112 NPS positive cases: 0.97% in 2012, 1.11% in 2013 and 3.69% in 2014. But most of the relevant cases concerned not only driving under the influence cases, but also cases possession of drugs, cases of intoxication, including fatal intoxication [10].

In summary, according to the scientific literature (from general population surveys and depending of the age), the prevalence of NPS use in the last year can be estimated between 0.1 and 3% in Belgium. Our data suggest that in a drugged driving population the prevalence of NPS might be higher.

It is obvious that consumption of NPS can lead to impairment similar to typical performance deficits caused by traditionally DOA use which are not compatible with safe driving [18]. In this way, agencies and toxicology laboratories involved in the investigation of alleged DUI cases can no longer just rely on analytical protocols that involve alcohol and classical DOA. But the main difficulty consists in NPS non-detectability by commonly used drug screening tests based on antibodies. A defective and questionable solution could be to perform a blood sampling (with subsequent implementation of screening techniques for NPS) in case of negative roadside tests and concomitant significant signs of impairment. More broadly, the difficulties experienced in (1) police, physicians as well as forensic toxicologists who should become familiar with the effects of NPS, (2) sophisticated screening procedures covering the complete range of available compounds or their metabolites, which have to be developed for blood, serum, urine or oral fluid testing, in agreement with the various legal approaches concerning regulation of DUID, and (3) the considerations that should be taken into account for the development of policy about this problem which are difficult to assess: cost/benefit ratio, prevalence of NPS in a driving population, implementation of an exhaustive detection of all the NPS positive cases.

Conclusion

The NPS prevalence of 5% observed according to these preliminary results should be confirmed and reassessed (in all the 556 blood samples). Prevalence data of different studies are difficult to compare. Ideally, the evolution of NPS use should be followed, year after year, in a similar population with a similar methodology and analytical process. Nevertheless, this observed significant value confirms the efficiency of the developed analytical approach for NPS screening [22] and the reality of NPS use in the driving population, even if they are already positive for classical drugs.

In all cases, these preliminary results confirm that while DUID controls work perfectly to diminish risk and create a deterrent approach in Belgium, a systematic toxicological screening for NPS would broaden the impairment picture in cases of traffic accidents.

Disclosure of interest

The authors declare that they have no competing interest.

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Toxicologie Analytique & Clinique (2017) **29**, 47—56

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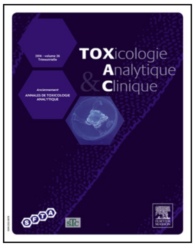

ORIGINAL ARTICLE

CHEMSEX, NPS & risk reduction management: Preliminary results of a pilot study

CHEMSEX, NPS et réduction des risques et des dommages : résultats préliminaires d'une étude pilote

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Received 31 October 2016; received in revised form 9 December 2016; accepted 9 December 2016
Available online 10 January 2017

KEYWORDS
Chemsex;
Harm reduction;

Summary ''Chemsex'' means sex, generally between men, occurring under the influence of drugs taken immediately before and/or during the sexual session. ''Slam'' is defined by injection of psychostimulant drugs in the same context. The Parisian Addictovigilance Center presents preliminary results of a specific harm reduction program for slammers and chemsexers, based

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http://dx.doi.org/10.1016/j.toxac.2016.12.003
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Cathinones;
LC-HRMS;
NMR

on the analysis of new psychoactive substances (NPS). Study was built by stages: (1) questionnaire of evaluation on chemsex practice, (2) toxicological analysis of collected drugs samples, (3) communication of results during Focus Groups with impact assessment. From January to September 2016, 17 questionnaires on chemsex practice and 31 samples were collected. Fifteen participants out of 17 were slammers. Motivations were mainly disinhibition and seeking pleasure (14 mentions) or prolonging pleasure (11 mentions). Collected samples were presented as cathinones (alpha-PVP, 4F-PVP, PVP-8, 3-MMC, 2-MMC, 4-MEC, methylone, MDPV), other phenethylamines (2-FMA, methamphetamine, 3,4-CTMP, 3-FPM) and cocaine. Analytical results show a 90% adequacy between the announced substance and the identified molecule. The Focus Group revealed a final level of knowledge on the harm reduction questionnaire at 9.4 on a scale of 12 items with a positive impact on: opinion on sample analysis (+ 1 point at the end of the session) and knowledge of the products (+ 0.6 points). This study is still ongoing and the final results should allow the implementation in routine practice of a validated approach of harm reduction in this specific population.

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Introduction

Chemsex (or Party and Play) is commonly understood to describe sex between men that occurs under the influence of drugs taken immediately preceding and/or during the sexual session [1]. The slam, considered as the hard part of chemsex is, as for it, characterized by the administration of psychostimulant drugs by intravenous injection. Such phenomena have particularly increased with the emergence of the new psychoactive substances (NPS) on the drug market [2].

In 2012, AIDES association (HIV/AIDS organization in France) and its partners carried out the first qualitative study among slammers and health care providers who are in contact with this population. Besides the marginal slam phenomenon, the report highlighted the wider phenomenon of consumption of NPS linked to sexuality between men. Cathinones were the most frequently reported products. Available on the Internet, renowned as pure, their users were unaware of their toxic and addictive potential. Moreover, chemsexers did not consider the risk of their practice, didn't identify with usual drug addicts and they had difficulties turning to centers dedicated to drug users [3].

In 2014, the Parisian Addictovigilance Center (CEIP-A) conducted a previous study, to establish the link between psychoactive substances (PAS) use and sexuality in a population of men who have sex with men (MSM). Cathinones, the main class of quoted NPS with synthetic cannabinoids, represented 29% of the declared consumptions during the last 6 months. Bought on the Internet, many of users usually did not have much knowledge about toxicity or risk reduction measures when they have started the slam practice. Moreover, they usually did not go to specialized structures as CAARUDs (Centres d'Accueil et d'Accompagnement à la Réduction des Risques pour Usagers de Drogues, the Support Centres for the Reduction of Drug-related Harms) because they often did not identify as drug users [4].

As a continuation to this first work, we want to develop and validate a specific harm reduction program for slammers and more globally for all chemsex participants. It is based on the toxicological analysis of NPS they consumed. Secondary objectives are to characterize NPS used in Party and Play, and to draw the profile of Parisian chemsexers/slammers. We aim to present the preliminary results of this project.

Methods

Study design

This study in adult chemsexers was conducted, through the course of 2016, in collaboration with Marmottan Hospital, Montevideo clinic and CAARUD AIDES — Les Halles; and based on 3 different stages ''(1), (2) and (3)'' as displayed in Fig. 1. The first stage is the evaluation of chemsex practices and NPS used in this context (1). The second part is a blind qualitative and quantitative analysis of the collected samples, performed by the toxicology laboratory at the University Hospital of Lille (2). In the last stage, results are communicated to users during a Focus Group with impact assessment on their knowledge and future practice (3). The French consultative committee for the protection of persons was consulted for the ethical angle. Simplified notification was made to the CNIL (Commission Nationale de l'Informatique et des Libertés, the French data protection authority), according to the compliance commitment to MR-003. The detention of controlled substances was supervised by the National Detection System of Drugs and Toxic Substances — SINTES (Système d'Identification National des Toxiques et Substances) directed by the French Monitoring Centre for Drugs and Drug Addiction — OFDT (Observatoire Français des Drogues et des Toxicomanies). A month-long promotion of the study was made in both medical structures and CAARUD by partners. Flyers were distributed in all sites. In addition, promotion was made on a specific gay website.

Participants

For products analysis

Eligible participants are users of chemsex, older than 18 years, frequenting the CAARUD AIDES — Les Halles or

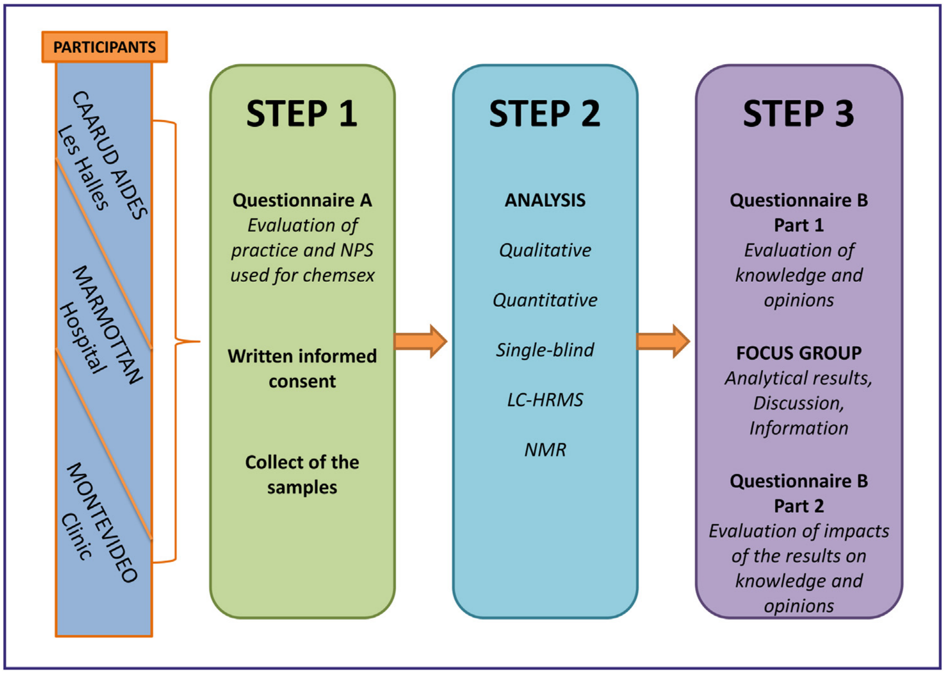


Figure 1. Workflow of the study.

consulting medical services, and having filled out the written informed consent.

For the Focus Group session

Participants were recruited in the Paris agglomeration through medical consultation and/or distribution of flyers on the Internet and/or in the CAARUD. The public was consequently wider than for the stage of samples' collection. The study was presented as a Focus Group study without inclusion restriction and where everybody could give freely his opinion. It was explained that participation would be voluntary, all information provided by the participants would be confidential, and their individual-related data would not be linked with the recorded data of the project.

Samples collection and questionnaire on their consumptions

This first step was made during medical consultation or in the support center. Participants had to fill out a prior consent before completing a questionnaire A. Each user brought the quantity of product he wished (preferably in the original packaging). Collection was made employing an anonymous reference, later used to give back the analytical results and personal information during a second individual interview.

The questionnaire A is a not validated questionnaire, created by the CEIP-A, which aimed to evaluate consumptions of slammers and chemsexers. It was based on various items as follows: profile of Party and Play participants (*How long did you use NPS in a sexual context? What is the frequency of use? The route of administration?*), NPS used in sexual context (*Did you have already tried the drug? Was it an advised product?*), intended effects *(pleasure, disinhibition, facilitation of penetration, delayed ejaculation, other*), details on obtainment method (*Internet or gift*) and criteria for their purchases on the Internet (*Which website? Which quantity? Which purchasing factors?*). Knowledge of toxicity and prevention was also evaluated (*method of injection, harm reduction tools*) as well as their opinion about toxicological analysis of the product as a harm reduction tool (*Agree or not? Which motivations? Which acceptable time for results?*). All these collected data were analyzed by descriptive biostatistics (descriptive statistics, univariate and multivariate analysis).

Analytical methods

After macroscopic description and grinding using mortar (if necessary), one 10 mg aliquot of each sample was solubilized in 1 mL of methanol, sonicated for 5 minutes, and subsequently 1:10 000 diluted in methanol: 100 μL of the obtained solution were evaporated to dryness using nitrogen. The obtained residue was reconstituted in 100 μL of the mobile phase (A/B; 60/40%) before analysis using liquid chromatography with high resolution mass spectrometry detection (LC-HRMS) with a UPLC-Xevo G2 QTOF (Waters) system. The chromatographic separation was performed into an Acquity TMHSS C18 column (1.8 μm, 2.1 × 150 mm) (Waters) with an oven temperature of 50 °C, using mobile phases including ammonium formate buffer 5 mM, pH 3 (A) and acetonitrile in 1% formic acid (B); flow rate of 0.4 mL/min was used. Initial concentration of mobile phase B (3%) held until 0.5 min, increased to 40% at 25 min, then to 100% at 26 min, and finally held until 30 min. Ionization was performed in positive electrospray mode: capillary voltage of 1 kV, source temperature at 140 °C, desolvation gas temperature of 420 °C and flow rate of 1000 L/h, 20 V as ion spray voltage. Conditions of acquisition were as follow: MS^E mode (acceleration energy of 5 eV, collision energy ramp from 10—40 eV, lockmass using a Leucine Enkephalin infusion at 2 ng/μL), 0.2 sec as time of scan and argon as collision gas. The LC-HRMS device is controlled by MassLynx™ 4.1 software and data process was performed using ChromaLynx, TargetLynx, MassFragment and MetaboLynx associated software (Waters) using a home database of more than 1400 substances including nearly 300 NPS or metabolites [5,6].

For some samples (when we didn't have the pure substance, in the goal of complete the identification by high resolution mass spectrometry for a formally identification of the NPS, and to assess the purity of the sample), LC-HRMS analysis was completed by nuclear magnetic resonance analysis (NMR) [6]. The NMR spectra were recorded on AVANCE 300 (Bruker Biospin, France) operating at 300 Mhz equipped with a 5 mm quadruple nucleus probe (QNP) probe at 295 K. The ^{1}H spectra were recorded with 64 scans, a 32-K time-domain data points with 4800 Hz spectral width, an acquisition time of 3.42 s, a relaxation delay of 2 s and a flip angle of 30°. The Free Induction Decays (FIDs) were transformed (0.3 Hz broadening) and the baseline was corrected. For quantification, 10 mg of sample were dissolved in 600 μL of methanol-D4 and ERETIC (Electronic REference To access In vivo Concentrations) method was used for the determination of absolute concentrations. ERETIC consisted of a digitally generated Gaussian peak (calibrated using a reference sample) which was inserted into the spectrum of a sample by the software after processing the FID. With ERETIC, the integral in the spectrum gives directly the concentration of protons [7]. Consequently, using ERETIC method, the purity of a powder sample can be assessing by NMR in the absence of available pure substance sample [6].

Focus Group assessment

It began immediately after all group members had completed the self-report questionnaire B part one. The questionnaire B is a not validated questionnaire, created by the CEIP-A and constituted by 2 equal parts: part 1 (completed before the Focus Group session) and part 2 (completed after the Focus Group session). Each part includes: a first exercise based on several potential thoughts on analysis (to circle when appropriate for the user) and a second true or false exercise on products. The aim of this questionnaire was to evaluate the impact of discussions on opinion and knowledge at the end of the Focus Group session. Did the results have an impact on their thinking? Did they learn things during the session? Questionnaire B is attached in appendices (Appendix A).

For this study, the Focus Group discussion was selected as a research methodology. It is a technique of interview of group, semi-structured, which allows to explore and to discover wide-ranging information on a targeted topic. It is a qualitative survey which has, for objective, a better understanding of opinions, motivations and behavior. Its interest lies in the dynamics of group generated by interactions, which favors expression and discussion of various points of view (with the limit imposed by potential leaders). In contrary of individual interviews, focus group has the advantage to add depth and breadth to the data.

All Focus Groups were monitored by moderators, accompanied by a note-taker and an observer. The note-taker wrote each participant's contribution to the discussions as well as his or her non-verbal behavior.

Focus group data were audio-recorded and were later transcribed verbatim prior analysis. Next, the moderator and the note-taker reviewed transcriptions and discussed areas of consensus and discrepancies. Discrepancies were discussed with the observer, who did not participate in moderation or data collection.

The Focus Group discussion has explored questions about chemsex via thoughts, opinions, feelings and experiences.

Questions progressed from general to more specific.

The open-ended questions have started with an initial ''engaging'' question (e.g. *What expectations do you have for the meeting?*)

The Metaplan® technique was applied to answer. Every participant was invited to write individually his answers on papers. Then, the moderator of the session collected the papers and read them loudly. With the group, he classified by themes and glued the papers on a paperboard. The purpose was, for the participants, to bring some representations which were then discussed [8].

After that, some questions were asked to structure the debate and point up approaches. First question was: *What do you know about cathinones?* Second question was: *What is your impression about the analytical results of collected samples?* Last question was: *What do you know about the toxicity of cathinones?* People exchanged their knowledge and experiences. At the end of each debate, a summary was made by the moderator of the session.

The Focus Group ended when all group members had completed the self-report questionnaire B part two.

As no previous Focus Group study has been performed on the specific harm reduction of chemsex users, an inductive thematic approach was therefore implemented to analyse the data, involving familiarisation with the data, an open-coding process and data interpretation in themes derived from identified codes. The NVivo® software (Version 11) was used to code and manage the qualitative data following the principle of inductive content analysis [9].

The results are presented in a mind map form. This learning technique is a multi-sensory tool that uses visuospatial orientation to improve the integration of information. Inspirited from the elliptical notes of Leonardo da Vinci in his workbooks, mind mapping was developed by Buzan and Buzan [10] and already applied in studies [11].

Results

From January 2016 to September 2016, 17 questionnaires of evaluation of chemsex practice and 31 samples of substances were collected. One Focus Group session was organized in April 2016 with 29 participants among whom 19 answered the questionnaire B of impact of the results. Other Focus Groups are expected for November 2016 and January 2017.

Samples collection and questionnaire on their consumptions ($n = 17$)

Among these 17 users, 12 have regular chemsex parties (> 1 per month) and only two of them are recent chemsexers (practice < 2 months). 15/17 are slammers specifically, using the intravenous injection. Intended effects are disinhibition and seeking pleasure (14 mentions), prolonging pleasure

Table 1 Criteria linked to Internet purchase reported by users.

Which molecule? (*n*)	Recommended (6), owner choice (6)
Which website? (*n*)	Referenced website (12), deepweb (0) Recommended website (4), already tried (7)
Which quantity? (*n*)	2 grams (1), 5 grams (5), 10 grams (4), 25 grams (2)
Which purchasing factor? (mentions)	Form (10), proposed quantity (8), announced purity (6), price (5), packaging (2), dosage (1)

(11 mentions), facilitation of penetration (6 mentions) and delay of ejaculation (4 mentions). At the time of the collect, 15/17 have already tried the product and 10 of them were satisfied by the effects.

Products were obtained as gifts (5) or on the Internet (12). Criteria linked to the Internet purchase are summarized in Table 1.

Among these 17 users, 3 don't know which product they have consumed (either bought on the internet and never tried before [2 cases] or done by a partner without any information [1 case]). Six declare to have no idea about the toxicity of the products but all slammers explain to have knowledge on injection practice and harm reduction. Motivations linked to products analysis are summarized in Fig. 2.

Toxicological analysis (*n* = 31)

Collected samples were presented as cathinones: Alpha-pyrrolidinovalerophenone or α-PVP (3), Fluoro-alpha-pyrrolidinovalerophenone or 4F-PVP (1), Alpha-pyrrolidinoheptiophenone or PVP-8 (1), 3-Methylmethcathinone or 3-MMC (8), 2-Methylmethcathinone or 2-MMC (1), 4-Methylethylcathinone or 4-MEC (8), methylone (1), Methylenedioxypyrovalerone or MDPV (1), other phenethylamines: 2-Fluoromethamphetamine or 2-FMA (2), methamphetamine (2), 3,4-Dichloromethylphenidate or 3,4-CTMP which is a methylphenidate analogue (1), 3-Fluorophenmetrazine or 3-FPM (1) and cocaine (1).

In this study, the home HRMS database used is of more than 1400 substances including nearly 300 NPS or metabolites [5,6]. Therefore, the LC-HRMS method, with NMR in addition, exhibits acceptable analytical criteria for drug sample identification regarding to literature recommendations, even if it take place in the challenging NPS area with an increasing and dynamic market [12].

Toxicological analysis showed a 90% adequacy between the announced product and the real drug in the sample. The variability of purity is high, as shown for 3-MMC and 4-MEC in Fig. 3. Ten other per cent (3 samples) consist in:

- not the announced product but the same chemical class (3-MMC sold for 4-MEC);
- not the right product and a different chemical class (5-MeO-DIPT, an hallucinogen tryptamine sold for α-PVP, a stimulant cathinone);
- no psychoactive substance at all (PVP-8).

Moreover, no pharmacologically active cutting agent was found. No specific organic substance was highlighted suggesting that cutting agents consist of mineral salts.

Focus Group session (*n* = 29)

The Focus Group participants are MSM (27) or women who have sex with women (2).

Analysis combined content and thematic; and focussed on participants' views of cathinones use for chemsex. The transcriptions of discussions were coded and classified in themes such as: *The How? The Why? The Risks?* and *The Emotional experiences* and sub-themes like: *Form*, *Toxicity*, *Stigmatization*, *Limits* as presented in Fig. 4.

The How

The participants were interested in the technical details of drug administration: intravenous and intrarectal route were the most cited. The different forms were approached with a real fascination to know the purest form.

The Risks

The complications quoted were focused on craving/dependence, comedown, and infectiology. There was little concern about poisoning (coma/death) or venous toxicity. On the contrary, chemical submission (or drug facilitated assault), and cutting agent toxicity appeared in the debate. A related and common concern was harm reduction.

The Why

The reasons why they used chemsex were related to the positive effects on sexuality and to the notion of ''challenge the limits'' and ''cross the line''.

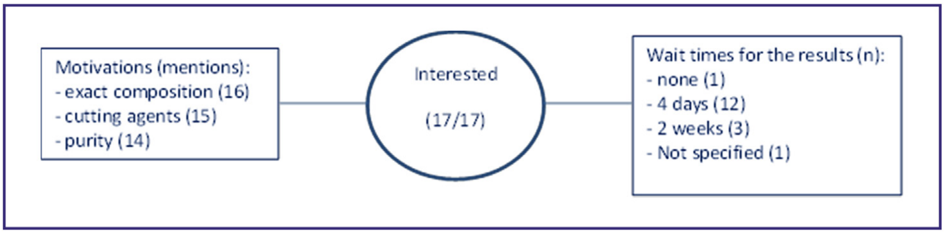
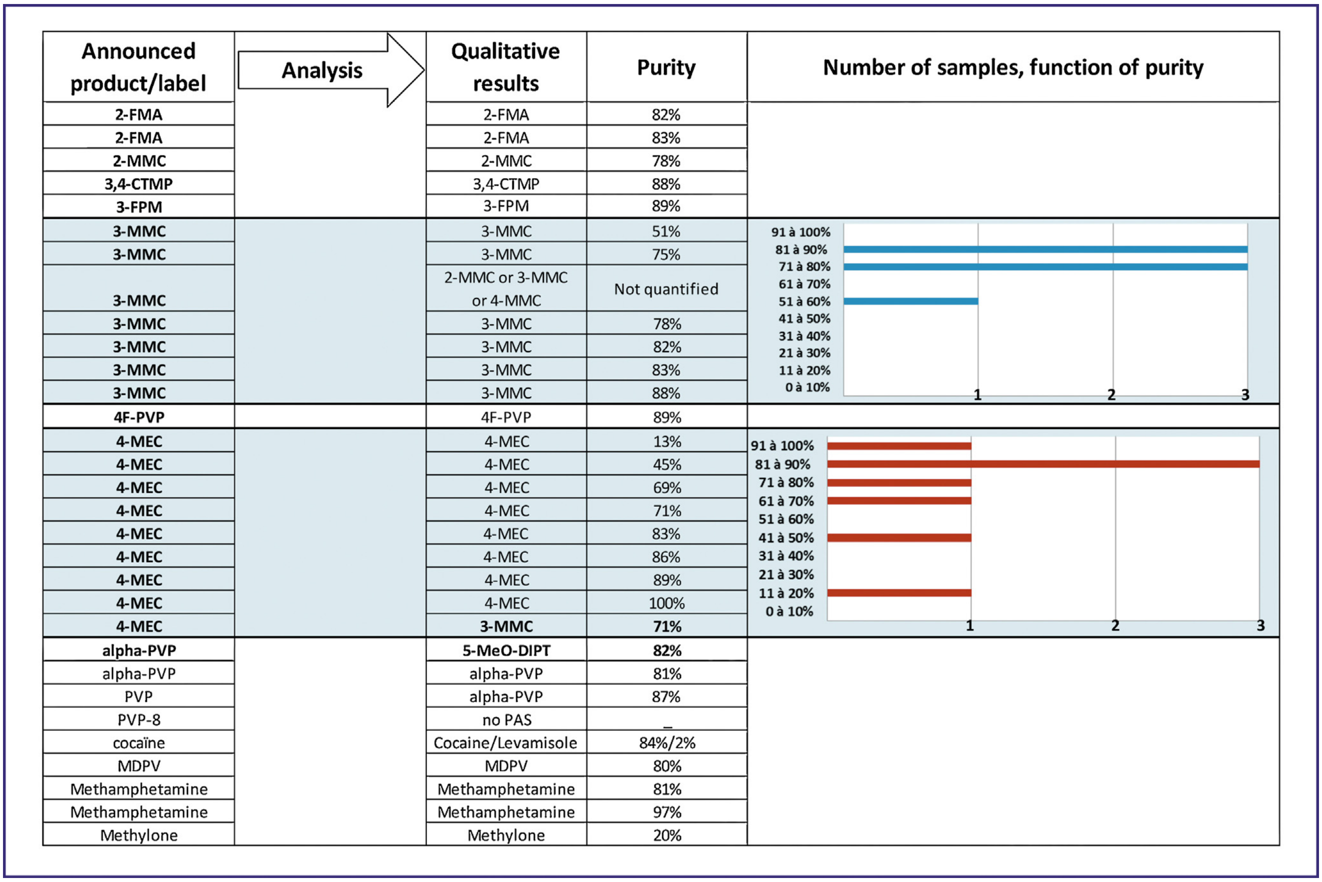


Figure 2. Motivations linked to products analysis reported by users.



Announced product/label	Analysis	Qualitative results	Purity	Number of samples, function of purity
2-FMA		2-FMA	82%	
2-FMA		2-FMA	83%	
2-MMC		2-MMC	78%	
3,4-CTMP		3,4-CTMP	88%	
3-FPM		3-FPM	89%	
3-MMC		3-MMC	51%	
3-MMC		3-MMC	75%	
3-MMC		2-MMC or 3-MMC or 4-MMC	Not quantified	
3-MMC		3-MMC	78%	
3-MMC		3-MMC	82%	
3-MMC		3-MMC	83%	
3-MMC		3-MMC	88%	
4F-PVP		4F-PVP	89%	
4-MEC		4-MEC	13%	
4-MEC		4-MEC	45%	
4-MEC		4-MEC	69%	
4-MEC		4-MEC	71%	
4-MEC		4-MEC	83%	
4-MEC		4-MEC	86%	
4-MEC		4-MEC	89%	
4-MEC		4-MEC	100%	
4-MEC		**3-MMC**	**71%**	
alpha-PVP		**5-MeO-DIPT**	**82%**	
alpha-PVP		alpha-PVP	81%	
PVP		alpha-PVP	87%	
PVP-8		no PAS	_	
cocaïne		Cocaine/Levamisole	84%/2%	
MDPV		MDPV	80%	
Methamphetamine		Methamphetamine	81%	
Methamphetamine		Methamphetamine	97%	
Methylone		Methylone	20%	

Figure 3. Analytical results for the 31 collected samples. 2-FMA: 2-Fluoromethamphetamine, 2-MMC: 2-Methylmethcathinone, 3,4-CTMP: 3,4-Dichloromethylphenidate, 3-FPM: 3-Fluorophenmetrazine, 3-MMC: 3-Methylmethcathinone, 4F-PVP: 4-Fluoro-alpha-pyrrolidinovalerophenone, 4-MEC: 4-Methylethylcathinone, PVP: Pyrrolidinovalerophenone Alpha-PVP: Alpha-pyrrolidinovalerophenone, PVP-8: Alpha-pyrrolidinoheptiophenone; 5-MeO-DIPT: 5-methoxy-diisopropyltryptamine, MDPV: Methylene-dioxypyrovalerone, PAS: psychoactive substance.

Emotional experiences

Participants expressed both a fascination and a stigmatization for the slam. The stigmatization of caregivers has been raised on several occasions, but also the stigmatization comes from the gay community. Positive and negative emotions were involved with drug use. Negative emotions focused on anxiety/fear, comedown and dissociative disorders. Positive emotions were evoked through the first flash effect (rush) and the sexual pleasure.

Some of the comments about these four themes are reported in Table 2.

Questionnaire B

Nineteen participants replied the questionnaire B (part 1 and 2). Concerning the 10 no-answer, various explanations can be given: users disagreed to complete the questionnaire, people were interested but not concerned by chemsex or people left the session with their questionnaire.

They had an average age of 47 ± 12 years. The majority of them used intravenous route (11/19). The questionnaire B has assessed the level of knowledge on harm reduction at 9.4 on a scale of 12 at the end of the session. An impact of the analysis results was determined by a change of opinion in 70% of the participants. Moreover, this first Focus Group showed an evolution, in a positive way, regarding opinion on sample analysis (+ 1 point at the end of the session) and knowledge on the products (+ 0.6 points).

Discussion

There is an increase of MSM and more globally gay, bisexual and other men who have sex with men (GBMSM) reporting chemsex sessions. However, there is currently a lack of published evidence for specific interventions dedicated to them [13,14]. Harm reduction programmes investigate various orientations and develop some tools but toxicological screening of the products is currently missing. In 2010, the collective expertise of Inserm (The French National Institute of Health and Medical Research) on risk reduction in drug users, already recommended an adaptation of the measures, according to the evolution of practices, profiles of users and context of consumptions. Furthermore, they underlined the importance to develop field studies for a better understanding of users' needs and to adapt the tools of risk reduction [15]. This study aims at promoting analysis as a new method, an innovative tool for a global program of harm reduction dedicated to chemsexers.

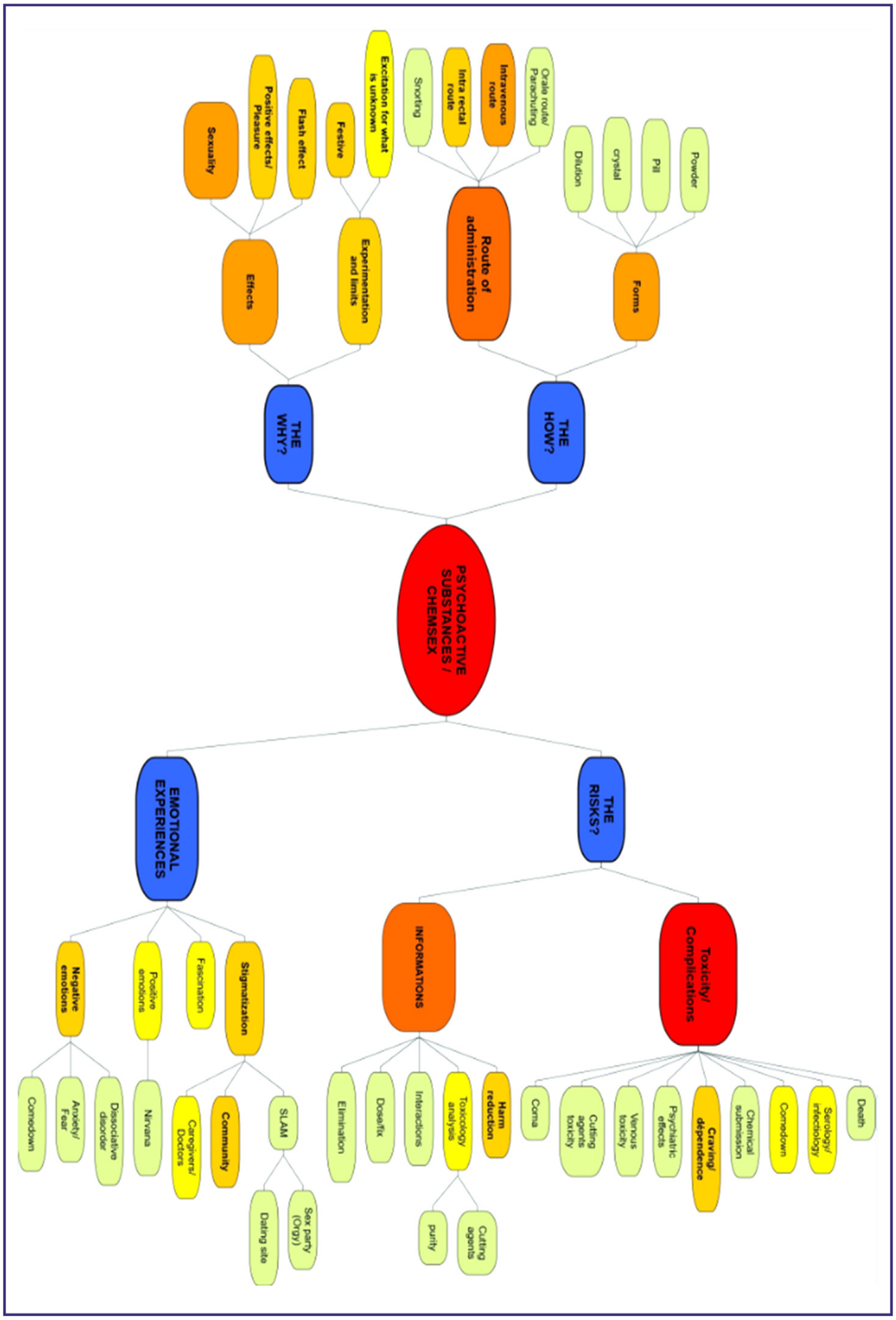


Figure 4. Mind map of the Focus Group session.

Key points

In a first point, it was essential to integrate GBMSM who use NPS into the elaboration of our harm reduction program, an involvement from the collection to the Focus Group. Mobilization was important. Different profiles of GBMSM drug users emerged but all were concerned by the same goal: to have sex under the influence of PAS. While the first step of the study concerned more particularly 15 men slammers, with a longer experience of slamming and already involved in harm reduction, the Focus Group drew, for its first session, 29 chemsexers, men and women. Moreover, education by the peers, sharing information, experiences and diffusion of harm prevention messages, has allowed the acquisition of new knowledge and skills. This program acts, for drug-users as a community and individual empowerment [16].

In a second point, analysis of the products seemed to meet a true need for the users. NPS arrived recently on the market and there are still numerous grey areas about them. Promotion of this study was made by flyers questioning about the confidence that chemsexers have in the

Table 2 Examples of statements made by Focus Groups participants.

Theme	Sub-theme	Examples of Statements Made by Focus Groups Participants
The how	Forms	*Does ''green pellets'' are Highest-Dose than powders??* *The crystal is purer than the powder which is mixed with cutting agent* *Taking a little G dose (GHB) before*
	Route of administration	*With rectal route, you don't have the same rush*
The risks	Toxicity/complications	*You can't seem to stop you, you have fallen further and further afield* *A doctor said you could stay in a high* (dissociative disorder)*!*
Emotional experiences	Stigmatization	*My doctor didn't know the ''slam'', so I did it* *I have friends who sniff but they stigmatize ''slammers''. We all have our different characters and approaches, with our private representations.*
	Positive emotions	*With my mixed of cathinones, it's the nirvana with no comedown...*
	Negative emotions	*They fail to prick, it's anxiety-provoking: It sucks!*
The why	Experimentation and limits	*I agreed to slamming without knowing what they offered me!* *I really accepted because everyone did it!* *Crossing the line is in the ''drugs'' and ''sex'' concept! It all goes together!*

substances they consume. In reality, they are relatively careful and warn their peers to distrust this kind of products. On the other hand, they relatively trust the ''old'' molecules as cocaine, owing to the ''*pseudosafety*'' of these substances of reference, gold standard for Party and Play.

As the third point, regarding to the results of toxicological analysis, it is undeniable that there is a strong adequacy between the announced product and the identified drug. But this is not always the case. And it is on this point that information and prevention messages were given and shared by participants at the Focus Group. Last statistical data from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in 2012 referred caffeine, lactose, cellulose as the most frequent substances reported as cutting agents of illicit drug tablets. However, three European countries seem to have some specificity; as in the Netherlands where piperazines have been detected in the composition of illicit drug tablets and other NPS have been used as adulterants for ecstasy or ''speed'' [17,18]. Users often have a preconceived idea that the NPS present high purity. Our results have shown that concentrations varied greatly as underlined with 4-MEC (min: 13%—max: 100%). Precaution is appropriate. One of our previous studies, dealing with analysis of NRG-3 samples, has already shown various qualitative and quantitative results [19].

Weak points

We present here the preliminary results of a pilot study for the first nine months. The toxicology laboratory of the University Hospital of Lille analyzed 31 samples. It's less than expected. We notice that it is quite difficult to obtain products from users. They actually have difficulty to give a little sample of their powder. For the end of the study, more follow-ups will be made with the partners to promote and disseminate the program.

Furthermore, the Focus Group allowed to discuss, in a group, on the global results of the analyzed samples. At the end of session, each user has the possibility to come, with his reference number, for a personalized depiction of the results. Few of them asked for more information. Users are favorable to the analysis of products but according to a rather short deadline of the results depiction (few days). Very often, products are already consumed at the time of the transmission of information.

What does the Focus Group teach us for our harm reduction program?

The variation of composition via sample analysis represents an interesting approach of harm reduction related to the use of chemsex. It's now recognised that harm reduction policy needs specific interventions into this MSM community on these two sources of risks: sexual behaviour and drug use [20]. Focus Group, which offers insights into the real worlds, experiences and perspectives of chemsex users can be considered as a good tool for sharing harm reduction counselling [21].

Some topics like drug-facilitated sexual assault (also called chemical submission), or drug-induced sexual vulnerability, have been raised. It's a real problem, already highlighted by Bourne et al. [14]. In their study, 3 out of 30 men had been victims of non-consensual sex (and several others mentioned they had witnessed sex where the issue of giving consent seemed problematic).

Participants seemed to be worried about the toxicity of adulterants than the toxicity of cathinones. Speaking about adulterants could be a first step to use a harm reduction approach with chemsex users.

Drug users concerns are different from caregivers concerns: craving/dependence, fear of dissociative disorder, infectiology and comedown are the most cited while the medical community reports that chemsex disclosure is associated with higher risk-taking behaviors, acute sexually transmitted infections, including hepatitis C incidence [22]. These concerns of participants identify possible ways of discussion with chemsex users in order to have the first interaction.

Consequently, one of our perspectives would be to create a ''mind map of reference'', for all the partners, around the problem of chemsex, to allow them a facilitated exchange and a global knowledge of the themes of interest.

Conclusion

It can be very difficult and possibly taboo for the population of GBMSM practicing chemsex to go to specific care structures and even more to ask for a medical psychological coverage. In a proactive approach, information, prevention and harm reduction, via a specialized process adapted to the chemsex, turn out relevant and necessary. The analysis of products, as a tool for harm reduction, shows a promising interest. Experience of first Focus Group showed a positive evolution regarding opinion on sample analysis. The study is still ongoing and the definitive results should allow the implementation in routine practice of a validated approach of harm reduction in this specific population.

Disclosure of interest

Dr Batel has conflicts of interest to disclose with Gilead, Ethypharm, D&A pharma, Abvie and Lundbeck.

The other authors declare that they have no competing interest.

Appendix A. Overview of the questionnaire B

Table 2 Exercise 1: Circle the sentences most relevant for you:.

Product analysis is not interesting, only effect is important.	It is important to know the results of analysis and to be able to discuss them after
I want to know the results, I don't need about information	It's important to know what we consume, and it could change my practice.
I'd rather not know what I consume	To talk about risks of the products could change my practice.
I would like to have the results of analysis before the consumption to know my risks	Analysis is an approach without judgment which suits me perfectly
If I have the results before the intake and if the real drug is not the announced product, anyway, I will experiment the product	Product analysis has no interest, I trust in the product I use.
If the product is pure, i twill be better (more effects)	If I have the results before the intake and if the product drug is not the intended drug, I won't experiment the product.
It's interesting, but it won't change my practice	To know adulterants, it's no use.
I would like to have the results of analysis. Before or after the intake, doesn't matter.	I prefer to know if there are adulterants.
Products I use are pure, I'm not afraid It is reassuring to be certain of what we consume	If the product is very pure, we'll have to be more careful (more effects, thus potentially dangerous)
I already inquire about risks and toxicity of the product before using it (opinion of other users, web research)	I don't want to give a sample of my product for analysis
I'm safe because I always begin to test a small quantity of product.	I have already wished to make analyze my products I already use harm reduction with my partners

Table 2 **Exercise 2: True or False.**

According to you:		
Cathinones are not drugs as cocaine, heroin	T ☐	F ☐
The composition of 4-MEC for example may vary from a retail website to another one	T ☐	F ☐
The composition of a product may vary on the same website and over time	T ☐	F ☐
If the products belong to the same chemical family, they will have the same effects	T ☐	F ☐
On the Internet, scams are rare	T ☐	F ☐
The more products are new, the more they are strong and safe	T ☐	F ☐
The dangerousness of products results largely from their purity and from their cutting agents	T ☐	F ☐
Cutting agents have no effect and no toxicity	T ☐	F ☐
The purity of products allows a stronger effect in complete safety	T ☐	F ☐
The purity of products is dangerous, it conditions the dose	T ☐	F ☐
It is always necessary to begin with a small quantity of product	T ☐	F ☐
To have some knowledge on products allows to check better the risks of my practice	T ☐	F ☐

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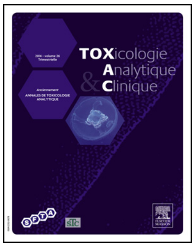

ORIGINAL ARTICLE

Characterization of metizolam, a designer benzodiazepine, in alternative biological specimens

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Received 29 July 2016; received in revised form 19 September 2016; accepted 19 September 2016
Available online 20 October 2016

KEYWORDS
Designer benzodiazepines;
Metizolam;
Alternative specimens;
Saliva;
Hair;
Exhaled breath;
Sweat

Summary Designer benzodiazepines provide an attractive alternative to prescribed benzodiazepines for abuse purposes as they are readily available over the Internet at a low price. Although these compounds have been developed by pharmaceutical companies, at this time, they do not have a legal status. Their detection in biological fluids is challenging owing to the very few available data. For instance, there is no information about metizolam (also known as desmethyletizolam) analysis, distribution and metabolism in the scientific literature. Metizolam was ordered via Internet and a 2 mg blue tablet was orally administered to a 54-year-old healthy man (85 kg). Oral fluid (saliva) was collected over 8 hours using the NeoSal^{TM} device. Sweat was collected with the PharmChek^{TM} sweat patch technology over 72 hours. Exhaled breath was collected with the ExaBreath® DrugTrap device over 15 hours. Beard hair was collected 4 and 10 days after administration. Finally, head hair was collected 3 weeks after administration. Metizolam was detectable in saliva during 8 hours, with concentrations lower than 1 ng/mL (max at 0.77 ng/mL after 2 hours). Increasing concentrations up to 186 pg/patch (after 72 hours) of metizolam were detected in the sweat patches. Metizolam produced for 4 hours a very low but significant chromatographic signal in exhaled breath, with concentrations always lower than 10 pg/filter. Finally, metizolam tested positive in beard hair (0.73 and 0.28 pg/mg after 4 and 10 days, respectively) and head hair (0.27 pg/mg). From the data obtained from one subject,

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http://dx.doi.org/10.1016/j.toxac.2016.09.004
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metizolam showed very low concentrations in various alternative matrices, attesting that the potential forensic implications (drug-facilitated crime, addiction, DUID...) of metizolam abuse must be considered as a new challenge for toxicologists.

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Introduction

Benzodiazepines are central nervous system depressants commonly prescribed to treat anxiety, insomnia, and seizures. Benzodiazepines are considered safer alternatives to barbiturates because they have fewer side effects and have a reduced risk of causing an overdose. However, benzodiazepine abuse is often associated with people taking benzodiazepines in combination with other controlled substances such as opiates, methadone, and cocaine. Benzodiazepines are taken in combination with opiates in particular because they enhance the euphoric effects of opiates; for other drugs, such as stimulants, benzodiazepines may temper the negative side effects (e.g., restlessness, agitation).

Although not approved for use, new benzodiazepines have been observed in forensic cases (death, acute intoxication, drug-facilitated crime, driving under the influence of drug). This includes phenazepam [1,2], etizolam and pyrazolam [3], flubromazolam [4], or clonazolam, meclonazepam and nifoxipam [5].

The detection of designer benzodiazepines in biological fluids is challenging owing to the very few available data. Some of these drugs have been detected using immunoassays with high cross-reactivity [3,6], demonstrating that designer benzodiazepines can be detected in standard blood or urine immunoassay drug screening. Other authors have developed liquid chromatography mass spectrometry methods, mostly to study the metabolism of specific drugs, including clonazolam, meclonazepam and nifoxipam [5], pyrazolam [7], flubromazepam [8], diclazepam [9], and clonazolam, deschloroetizolam, flubromazolam and meclonazepam [10]. Among the designer benzodiazepines group, the thienodiazepines, obtained after replacement of the benzene ring by a thiophene ring, are represented by etizolam (licenced for use in Japan, Italy and India), and more recently by metizolam (Fig. 1), also known as desmethyletizolam. Metizolam differs structurally from etizolam through removal of the methyl group on the triazole ring. Although etizolam has already been described in overdose [11] and death cases [12], there is no citation for metizolam (PubMed, consultation on 24 July 2016). For instance, there is no data about metizolam analysis in alternative biological specimens, such as hair, saliva or exhaled breath. This compound was patented by a Japanese company in 1995, as an anti-anxiety medication. It has no history of human usage prior to its availability by online vendors in September 2015.

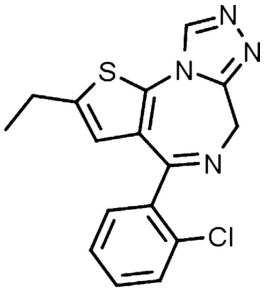

Figure 1. Chemical structure of metizolam.

A UPLC-MS/MS technique were used to detect metizolam in saliva, hair, sweat and exhaled breath after administration of an oral dose of 2 mg to a human volunteer.

Material and method

Self-administration study

To gain preliminary data on the detectability of metizolam in various alternative specimens, one of the authors (male, 54-year-old, 85 kg) ingested one blue tablet (declared 2 mg metizolam) with 200 mL of tap water. Oral fluid was collected over 8 hours using the NeoSal^{TM} (Neogen) device and used as recommended by the manufacturer, by handling the pad in the mouth of the subject for 2 min. Immediately thereafter, the pad was placed into the diluent, present in a plastic tube. Sweat was collected with the PharmChek^{TM} (Sudormed) sweat patch technology over 72 hours. Four patches were placed over the skin of the right arm at T0 and periodically removed. Exhaled breath was collected with the ExaBreath® DrugTrap Sensa Bues^{TM} device (Sensa Bues^{TM}) over 15 hours. This device consists of a filter that traps the aerosols (that contain the drugs) from breath. The test was complete after 20 breaths through the device, which approximately corresponds to 20—30 litres of breath going through the filter (about 2 min). No mouth rinsing was performed prior to breath sampling. Beard hair was collected 4 and 10 days after administration. Finally, head hair was collected 3 weeks after administration. Except hair, all specimens were stored at +4 °C until analysis.

In France, approval by an ethics committee is not required for self-experiments (except for drugs of abuse, that is not the case for metizolam).

No blood sample was collected, as this requires specific authorizations due to the invasive nature of this specimen.

For identification and characterization of metizolam, nuclear magnetic resonance (NMR) spectroscopy was applied. No significant impurity was detected in the metizolam tablet using UHPLC-Q-TOF/MS or NMR (Fig. 2). In addition, the dosage of one tablet, using the ERETIC (Electronic REference To access In vivo Concentrations) method, revealed a dosage of 1.8 mg of metizolam, in agreement with the announced 2 mg dosage [13].

Chemicals and reagents

Metizolam (4-(2-chlorophenyl)-2-ethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a] [1,4] diazepine; CAS No. 40054-68-0) was obtained from Chemicalwire (London, UK).

Ammonium formate and formic acid were provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France). LC-MS grade water and acetonitrile were purchased from Biosolve (Dieuze, France), while methanol HPLC grade and 30% hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Diazepam-d_5 was purchased from LGC Promochem (Molsheim, France) and diluted to appropriate concentration (variable according to the matrix) using methanol.

ExaBreath® DrugTrap (SensAbues®), NeoSal™ (Neogen), and PharmChek™ (Sudormed) devices were kindly provided by the respective companies.

Saliva extraction

Metizolam was extracted from 1 mL mixed saliva and diluent in presence of 1 ng of diazepam-d_5 used as internal standard by 1 mL saturated ammonium chloride pH 9.5 buffer (adjusted with ammonia water) and 5 mL of dichloromethane/*n*-heptane/isopropanol (25/65/10, v/v). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 50 μL of acetonitrile/formic acid 0.1% (50/50, v/v).

It was not possible to evaluate the amount of collected oral fluid, even by weighing the test tube before and after the centrifugation, as the volume of the buffer and oral fluid varies from device to device.

Sweat extraction

The sweat pad was extracted with 5 mL of methanol in the presence of 1 ng of diazepam-d_5 (internal standard) by horizontal shaking for 15 min. After centrifugation, the methanol was evaporated to dryness (gentile nitrogen stream) and finally, the residue was solubilized in 50 μL of acetonitrile/formic acid 0.1% (50/50, v/v).

Exhaled breath extraction

The extraction procedure was done by putting the device with outlet down on top of a disposable glass test tube with the filter still in the device. Ten millilitres of methanol were added by pipetting it carefully onto the filter avoiding the wall. This was repeated 10 times with the same methanolic solution, in order to extract maximum of the drug. 1 ng of diazepam-d_5 was added to the methanol at the last step, as internal standard. After centrifugation, the methanol was evaporated to dryness (gentle nitrogen stream) and finally, the residue was solubilized in 50 μL of acetonitrile/formic acid 0.1% (50/50, v/v).

Hair extraction

Metizolam was extracted from 20 mg of hair in presence of 1 ng of diazepam-d_5 used as internal standard by overnight incubation in 1 mL saturated ammonium chloride pH 9.5 buffer (adjusted with ammonia water). Then, 5 mL of dichloromethane/*n*-heptane/isopropanol (25/65/10, v/v) were added. After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 50 μL of acetonitrile/formic acid 0.1% (50/50, v/v).

UPLC-MS/MS analysis

LC was performed using a Waters Acquity system. Chromatography was achieved using a Acquity BEH C18 column (100 × 2.1 mm, 1.7 μm) eluted with a gradient delivered at a flow rate of 0.5 mL/min (from 5% acetonitrile—95% formic acid 0.1% to a ratio 95—5% at 5.5 min). An injection volume of 10 μL was used in all cases. A Quattro Premier XE triple-quadrupole mass spectrometer (Micromass-Waters) was used for analyses. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of metizolam and the IS: capillary voltage, 1.0 kV; source block temperature, 120 °C; and desolvatation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 800 L/h. In order to establish appropriate multiple reaction monitoring (MRM) conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation (CID) of both species was performed. Collision gas (argon) pressure was maintained at 3.0×10^{-3} Bar and the collision energy (eV) was adjusted to optimize the signal for the 2 most abundant product ions: transitions m/z 328.9 to 275.0 and m/z 328.9 to 300.0 for metizolam. Transition m/z 328.9 to 275.0 was used for quantitation. The transition m/z 290.0 to 198.1 was used for diazepam-d_5. MassLynx 4.1 software was used for quantitation.

Because of the nature of the study (detectability of metizolam in alternative specimens), no full analytical validation was achieved. Linearity (6 points, correlation coefficient > 0.99) was observed for metizolam concentrations ranging from 50 to 1000 pg/mL, 0.1 to 10 pg/mg, 5 to 500 pg/patch and 5 to 50 pg/filter in saliva, hair, sweat and exhaled air, respectively. Within-batch precisions ($n = 6$) at 100 pg/mL (saliva), 1 pg/mg (hair), 50 pg/patch (sweat), and 10 pg/filter (exhaled breath) were better than 25%. The limits of quantitation were estimated to be 2 pg/mL, 0.1 pg/mg, 5 pg/patch and 5 pg/filter for saliva, hair, sweat and exhaled air, respectively, with a S/N ratio of 3. Under the chromatographic conditions used, there was no interference with the analytes by chemicals or any extractable endogenous materials present in the various matrices (matrix effect < 20%).

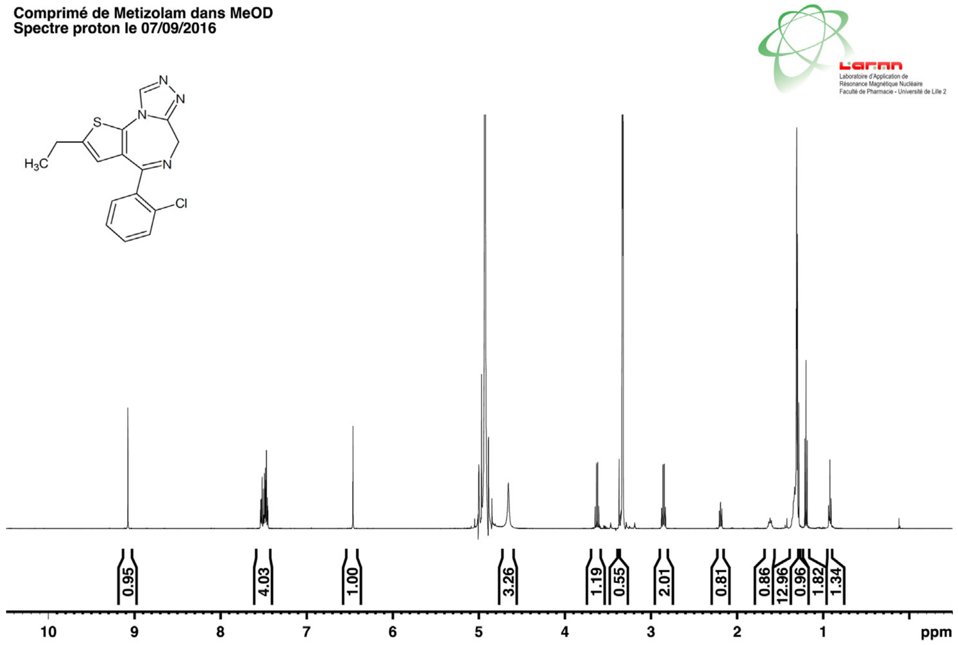

Figure 2. NMR analysis of the metizolam tablet.

Results and discussion

It is generally accepted that chemical testing of biological fluids is the most objective means of diagnosis of drug use. The presence of a drug analyte in a biological specimen can be used as evidence of recent exposure. The standard in drug testing is the immunoassay screening, followed by the gas or liquid chromatographic-mass spectrometric (GLC/MS or MS-MS) confirmation conducted on a urine sample. More recently, a variety of body specimens other than urine, such as oral fluid, sweat or hair have been proposed to document drug exposure.

When phenazepam and etizolam became scheduled in several countries, some laboratories turned their attention to investigational drugs, never before marketed as pharmaceuticals. This led to the appearance of designer benzodiazepines in Internet shops, sold for recreational purposes. A large series of drugs, including pyrazolam, flubromazepam, diclazepam, nifoxipam, meclonazepam or flubromazolam were available on the dark market. Among these new designer benzodiazepines, not much attention was given to metizolam. Besides the historical archives from Japan, there is a real dearth of literature on this drug. It was postulated the likely effects based on structural similarity to classic benzodiazepines, but the only available source of information is that which can be found on drug discussion forums.

After oral administration of 2 mg of metizolam to a 54-year-old healthy man (85 kg), which corresponds to a usual dose, the overall behaviour of the subject was not modified. A slight sensation of sedation was noticed, but impairment was not observed.

Metizolam was detectable in saliva during 8 hours, with concentrations always lower than 1 ng/mL. The Cmax was 0.77 ng/mL, obtained 2 hours after administration. The excretion pattern of metizolam is presented in Fig. 3. A typical saliva chromatogram is presented in Fig. 4. As it was described for other benzodiazepines [14], the excretion of metizolam in saliva was very weak. This should prevent its detection during routine analyses. Contamination of the oral cavity during the collection of the specimen after 30 min cannot be ruled out. The NeoSal™ (Neogen) was found suitable for saliva collection. The effect of collection methods on drug concentrations in oral fluid is not well described in the scientific literature. However, caution has to be taken. As the oral fluid flow rate increases (that can be the case when using a collection device), the concentration of bicarbonate ions increases. This may alter the saliva-plasma ratio in a pH-dependent manner, especially for weakly basic drugs with a pK_a approaching the oral fluid pH.

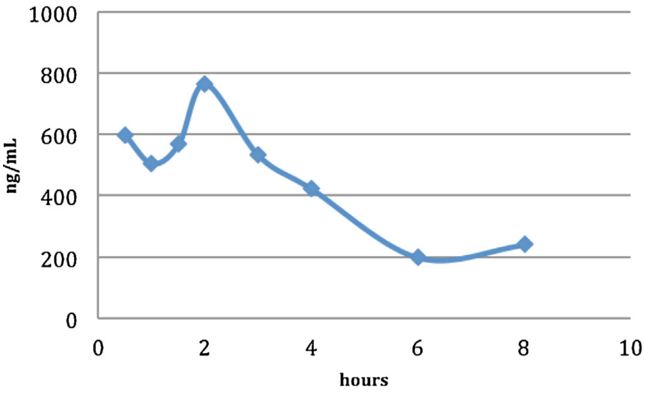

Figure 3. Concentration time profile of metizolam excretion in saliva after administration of a single 2 mg tablet.

Between 300 to 700 mL/day of *insensible* sweat is produced over the whole body, likely caused by diffusion through the skin, whereas 2 to 4 L/h of *sensible* sweat may be produced for short periods by extensive exercise. The amount of sweat that is produced is thus affected by body

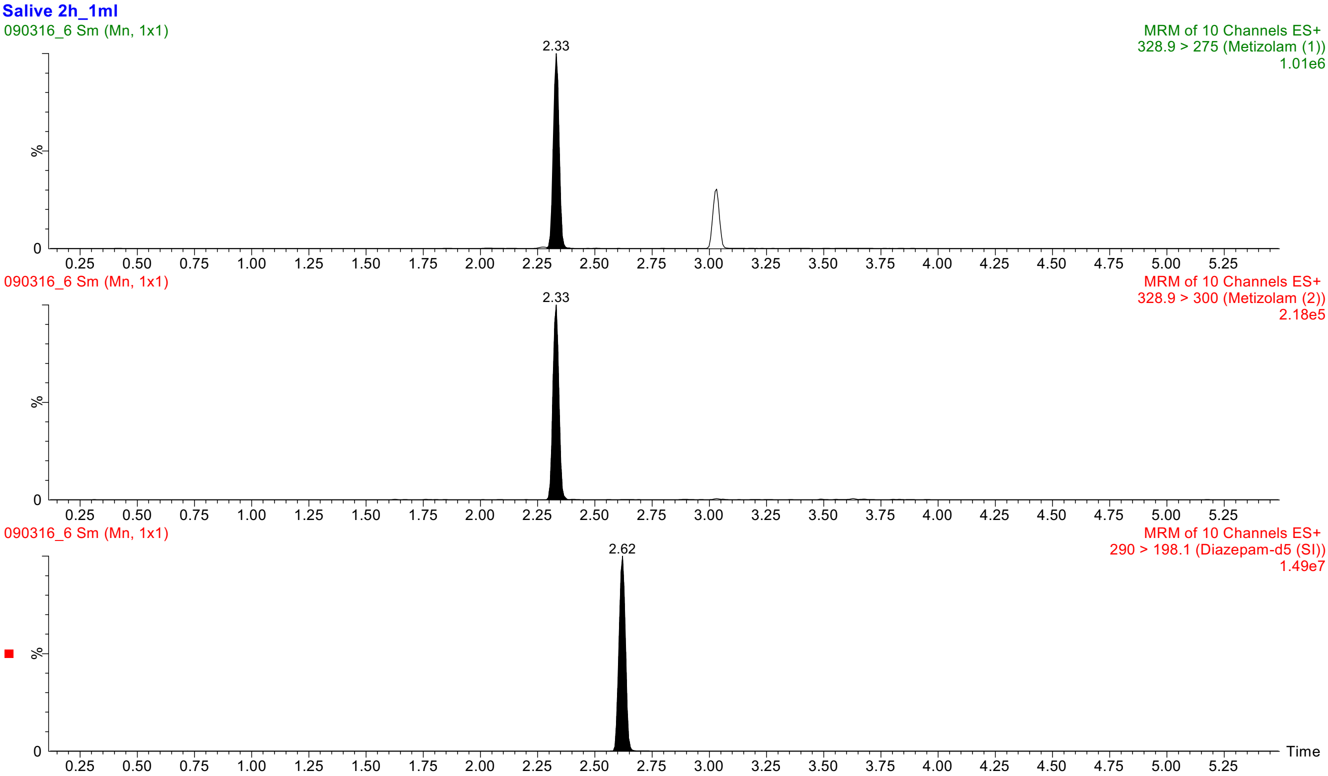

Figure 4. Chromatogram obtained in saliva collected 2 hours after oral administration of 2 mg of metizolam. Metizolam concentration was 766 pg/mL. From top to bottom: 2 transitions for metizolam and 1 transition for diazepam-d_5.

location, ambient temperature, body temperature and relative humidity of the environment and by emotional, mental and physical stress. The variability of these factors and the uneven distribution of the sweat glands make it difficult to obtain specimens of sweat systematically. Researchers have known since more than a century that drugs are excreted in sweat. Although it is still poorly understood how non-volatile chemicals exit the body through the skin, significant advances in sweat analyses have been made during the past years with the development of the sweat patch technology. The device consists of an adhesive layer on a thin transparent film of surgical dressing to which a rectangular absorbent pad is attached. The sweat patch acts as a specimen container for non-volatile and liquid components of sweat, including drugs. Non-volatile substances from the environment cannot penetrate the transparent film, which is a semi-permeable membrane over the pad that allows oxygen, water, and carbon dioxide to pass through the patch, leaving the skin underneath healthy. Over a period of several days, sweat will saturate the pad located in the centre of the patch and will slowly concentrate, and drugs present in the perspiration fluid will be retained. Sweat appears to offer the advantage of being a non-invasive means of obtaining a cumulative estimate of drug exposure over one week. Sweat testing has found applications in monitoring of individuals in drug rehabilitation or in probation/parole programs. Subjects can wear one patch with minimal discomfort for at least one week. Normal hygiene practices can be achieved. Attempts to remove the patch prematurely are readily visible to personnel trained to monitor the sweat patch [15]. Increasing concentrations up to 186 pg/patch (after 72 hours) of metizolam were detected in the sweat patches. The cumulative concentrations time profile of excretion is given in Fig. 5. As it is the case with other benzodiazepines, the concentration of metizolam was week [15].

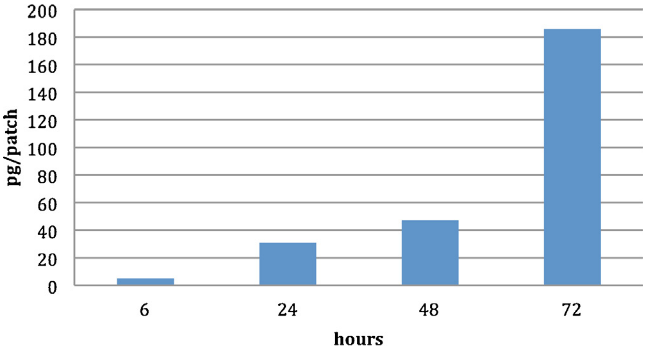

Figure 5. Cumulative concentrations time profile of metizolam excretion in sweat after administration of a single 2 mg tablet.

Until recently, exhaled breath was not considered to be a useful matrix for detecting drugs of abuse, despite some interest from the enforcement authorities to have a rapid tool to detect exposure, as is the case for alcohol. Exhaled breath was generally considered delicate to collect and analyse. A new tool, Drug Trap ExaBreath® from SensAbues® has recently been put onto the market and some applications have been published in the literature [16—19]. In comparison with blood or urine, collection of exhaled breath is less invasive, easier to control by medical staff or police officers, and

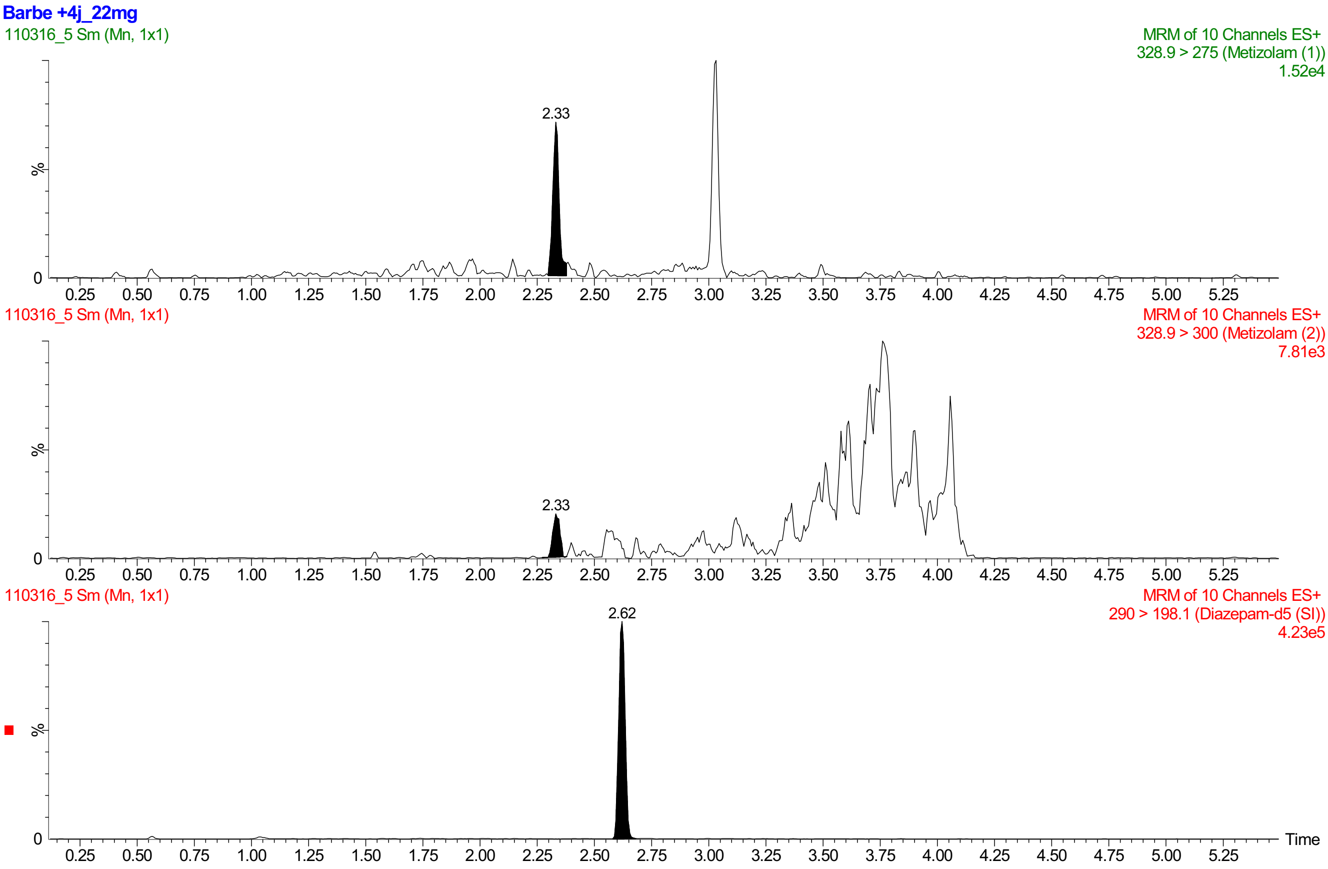

Figure 6. Chromatogram obtained in beard hair collected 4 days after oral administration of 2 mg of metizolam. Metizolam concentration was 0.73 pg/mg. From top to bottom: 2 transitions for metizolam and 1 transition for diazepam-d_5.

systematic collection can be completed for epidemiological studies. The collection device is easy to store and to ship to the laboratory. It can provide instantaneous information about a subject's exposure to substance(s). Metizolam produced for 4 hours a very low but significant chromatographic signal in exhaled breath, with concentrations always lower than 10 pg/filter. Data are presented in Table 1.

Finally, metizolam tested positive in beard hair (0.73 and 0.28 pg/mg after 4 and 10 days, respectively) and head hair collected 3 weeks after exposure (0.27 pg/mg). These concentrations are very low, in the same rage or even lower than classic benzodiazepines after a single dose [20]. The chromatogram obtained after beard extraction is presented in Fig. 6.

Table 1 Metizolam concentrations (pg/filter) in exhaled breath after oral administration of 2 mg.

Collection time after administration	Metizolam concentration (pg/filter)
0	Not detected
30 min	7
1 hour	9
90 min	6
2 hours	7
4 hours	5
6 hours	Not detected
15 hours	Not detected

Conclusion

It appears that the value of alternative specimens analysis for the identification of drug users is steadily gaining recognition. This can be seen from its growing use in pre-employment screening, in forensic sciences and in clinical applications. Saliva will probably be used in the next future in on-site testing both for epidemiological and screening purposes. It is extensively used in many countries for roadside safety. Hair analysis may be a useful adjunct to conventional drug testing in toxicology. Methods for evading urinalysis do not affect hair analysis. Alternative specimens can be more easily obtained with less embarrassment than for urine, and hair can provide a more accurate history of drug use. However, costs are too expensive for routine use but the generated data are extremely helpful to document positive urine cases. These new technologies may find useful applications in the next future, for example in doping control or law enforcement agencies to document illicit drug use.

After designer amphetamines, cathinones, synthetic cannabinoids, designer opiates (including fentanyl derivatives), the emergence of designer benzodiazepines is in the logical step on the market for ''legal highs''. After putting both phenazepam and etizolam on the list of controlled substances, there is space for new candidates from pharmaceutical research. Metizolam (also known as desmethyletizolam) is a short-acting psychoactive drug of the thienodiazepine class (the benzene ring has been replaced by a thiophene ring). It has been shown by anecdotal reports to produce depressants, anxiolytic, sedative, muscle relaxant and amnestic effects. Parent drug concentrations are very low in alternative matrices but detection is possible in the early hours after administration. The risk of missing metizolam during conventional screening is large, irrespective of the analytical technique used.

Except in UK (26 May 2016) and Sweden (26 January 2016), metizolam is not known to be specifically illegal. One major concern about this drug regarding public health is the lack of data on acute human toxicity and potential long-term effects.

Disclosure of interest

The authors declare that they have no competing interest.

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Toxicologie Analytique & Clinique (2017) **29**, 64—70

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ORIGINAL ARTICLE

Identification and quantification of diphenidine in hair by LC-MS/MS after single administration

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Received 12 September 2016; received in revised form 27 September 2016; accepted 27 September 2016
Available online 3 November 2016

KEYWORDS
Diphenidine;
New psychoactive substances;
Hair;
LC—MS/MS;
Dissociative drugs

Summary Diphenidine is a new psychoactive substance described by users as an anesthetic drug with mild stimulant potential and a large amnestic component. It appears to have been available since 2013, and was first detected in powders seized in 2014. Hair analysis allows distinction between acute or chronic drug consumption. Few data are available on diphenidine concentration in hair. We developed an analytical method in order to identify this compound in hair. After decontamination, 20 mg were grinded and incubated in phosphate buffer pH 5.0 in the presence of 100 ng of MDMA-d5 used as IS. Double basic liquid—liquid extraction was performed. Samples were separated on a 1.9 μm Hypersil GOLD PFP column (100 × 2.1 mm) using gradient elution. Compounds were detected by a LCQ TSQ Vantage XP triple-quadrupole mass spectrometer. The assay was accurate and precise over the range 10 pg/mg (lower limit of quantification) to 1000 pg/mg. A reproducible matrix effect around 40% was observed. We applied this method to a patient with a history of chronic drug abuse, who used diphenidine for the first time. He experienced dissociative effects, confusion, hallucinations, amnesia and severe temporal disorientation. A hair sample was obtained 49 days after ingestion, and was

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http://dx.doi.org/10.1016/j.toxac.2016.09.006
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divided in five 1 cm-segments. Diphenidine was identified on the first three proximal segments, probably due to sliding hair in the strand, at concentrations of 123, 79 and 89 pg/mg. Twenty-three others compounds were also identified in hair, showing the multidrug abuser status of this patient.

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Introduction

New psychoactive substances (NPS) are easily purchased on the Internet and are free of any legal restrictions in many countries. When restrictions do apply, emerging compounds are already available to replace such substances. Methoxetamine, which was first used as a ketamine substitute, has been restricted in France since August 2013. Some users have started looking for new dissociative compounds to replace methoxetamine. Diphenidine (1-[1,2-Diphenylethyl] piperidine or 1,2-DEP) and its related compound methoxyphenidine (1-[1-(2-methoxyphenyl)-2-phenylethyl] piperidine or MXP) are potential substitutes of methoxetamine and ketamine (Fig. 1). Diphenidine is a dissociative drug acting as a relatively selective NMDA receptor antagonist [1—3], with a weak inhibitory effect on dopamine and norepinephrine reuptake [3]. From a pharmacological point of view, it is less potent than other commonly dissociative drugs such as methoxetamine, PCP or ketamine [3]. In 2014, diphenidine was detected in seized powders in Italy [4] and also associated with synthetic cannabinoid in herbal products [5] or associated with other NPS [6]. There are few published cases of acute or chronic diphenidine exposure with or without analytical confirmation. Helander et al. [7] reported on a Swedish study over a 12-month period in which 750 cases of suspected NPS intoxication from emergency units were analysed, showing 14 non-fatal cases (1.9%) positive for diphenidine. Two fatal cases are known, but in both diphenidine was associated with others compounds [8,9]. Concerning the analytical methods available for the biological measurement of diphenidine, clinical publications usually did not describe the method used, and only one recent paper described the quantification in hair [10]. The purpose of this work was to develop and validate a method to detect and quantify diphenidine in hair, in order to confirm a case of poisoning with this compound in a hospitalized patient.

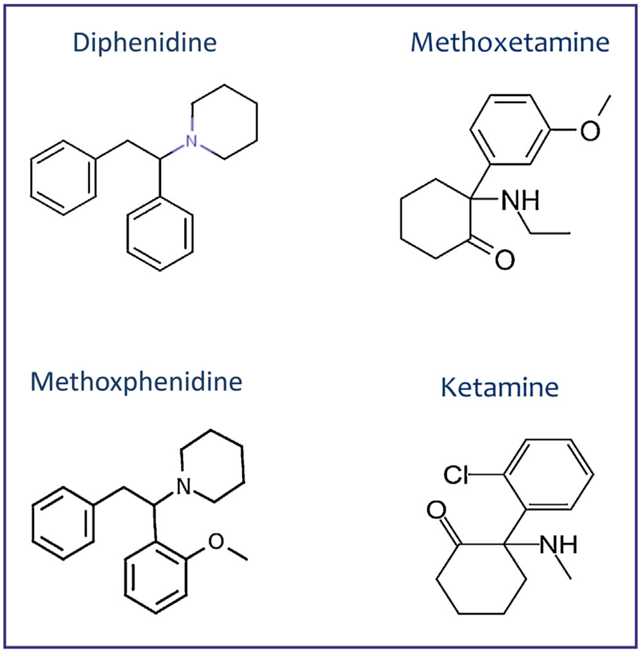

Figure 1. Chemical structures of diphenidine (1-(1,2-diphenylethyl)piperidine), methoxphenidine, methoxetamine and ketamine.

Experimental

Case report

A 19-year-old male was referred to the emergency unit, six hours after swallowing a powder wrapped in cigarette paper (''parachute''). He had a history of chronic drug abuse. He experienced difficulties purchasing methoxetamine online following legal restriction of methoxetamine in France. He bought diphenidine over the Internet for the first time, as a new drug expected to be a ''new legal methoxetamine replacement''. He did not use any other drug on that day and ingested an unknown amount of diphenidine at 11:00 p.m. (he bought 1 g but could not remember the amount ingested). He self-described dissociative effects with a marked temporal disorientation and hallucinations; he had no memory of most of this episode, until 8 hours later, when he woke up, still hallucinating. Six hours after ingestion, he was referred to the emergency unit of the local hospital, with confusion, disorientation, and hallucinations; Glasgow Coma Score was 13. His vital signs and electrocardiogram were normal until 11 hours after ingestion, when he suddenly developed anxiety and tachycardia with normal electrocardiogram, which resolved in response to clorazepate. His level of consciousness improved over the next hours and he was discharged to the psychiatric ward. A strand of brown hair was collected 49 days after the presumed unique ingestion. The length of the strand was 5 cm and was divided into 5 segments of 1 cm and 20 milligram each segment.

Chemicals and reagents

Acetonitrile, dichloromethane, hexane, ethyl acetate, formic acid and methanol were all high-performance liquid chromatography (HPLC) grade and were supplied by Sigma Aldrich (Paris, France). Diphenidine maleate was obtained at Tocris Bioscience (Bio-Techne, Lille, France) and MDMA-d5 (used as internal standard [IS]) purchased from LGC Standards (Molsheim, France). Sodium carbonate and sodium hydrogenocarbonate were from Prolabo (Paris, France). Ultra-pure water (18 MΩ) was obtained by ultrafiltration using a Direct-Q UV3 apparatus (Millipore Corp., Molsheim, France). Formate buffer (containing formate 2 mM and 0.1% formic acid) was prepared in ultra-pure water.

LC-MS/MS system and conditions

Chromatography was performed on Accela liquid chromatography system (Thermofisher, Les Ulis, France) coupled with a CTC analytics auto sampler (Thermofisher), using a 1.9 μm Hypersil GOLD PFP column (100 × 2.1 mm) maintained at 30 °C. The device was completed with a javelin filter (2.1 mm i.d.). The mobile phase was a gradient of acetonitrile and formate buffer (2 mmol/L formate in 0.1% formic acid) starting from 20% acetonitrile to 90% at the tenth minute at a flow rate of 300 μL/min. The total run time for an LC—MS/MS analysis was 12 min. Compounds were detected by a LCQ TSQ Vantage XP triple-quadrupole mass spectrometer (Thermofisher) equipped with an electrospray ionization (ESI) source. The ESI source was set in positive ionization mode, and an ion-spray voltage of +3.5 kV was applied. The heated capillary temperature was set at 350 °C. Nitrogen (Nitrox UHPLCMS 18, nitrogen generator, Domnick Hunter, Villefranche-sur-Saone, France) was employed as sheath and auxiliary gas at a pressure of 50 and 20 arbitrary units, respectively. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. Data were collected in selected reaction monitoring (SRM) mode, using the transition m/z 266.1 → m/z 103.1 (with a collision energy of 36 eV) and m/z 181.2 (19 eV) for diphenidine and m/z 199.1 → m/z 165.1 (12 eV) for the IS. Chromatographic data acquisition was performed using the Xcalibur software (v2.0.7 SP1, Thermofisher), and LCquan® software v2.5 was used for quantification.

Working solutions, calibration standard, and quality controls

Stock solution of diphenidine (1 mg/mL) and IS (100 μg/mL) were prepared in methanol. Working solutions of diphenidine for calibration standards (CS) were prepared at three concentrations (1, 0.1 and 0.01 μg/mL) by dilution of the stock solution in methanol. Working solution for quality control (QC) preparation was also prepared at the same concentrations by dilution in methanol of another 1 mg/mL stock solution prepared separately. Working solution of IS (0.2 μg/mL) was obtained by dilution of stock solution in methanol.

Table 1 Validation parameters of diphenidine in hair.

	Diphenidine
Linearity (pg/mg)	10—1000
LOD (pg/mg)	2
Correlation coefficient	> 0.996
Recovery (%)	
40 pg/mg	103.2
750 pg/mg	83.7
Mean matrix effect (%)	
40 pg/mg	37.6
750 pg/mg	40.3
QC 10 pg/mg	
CV intra-assay (%)	5.5
CV inter-assay (%)	9.3
Accuracy (%)	108.0
QC 40 pg/mg	
CV intra-assay (%)	7.0
CV inter-assay (%)	2.4
Accuracy (%)	102.7
QC 200 pg/mg	
CV intra-assay (%)	6.7
CV inter-assay (%)	13.1
Accuracy (%)	101.6
QC 750 pg/mg	
CV intra-assay (%)	7.6
CV inter-assay (%)	12.8
Accuracy (%)	102.3

Calibration curves were prepared by spiking with appropriate volumes of the previously mentioned working solutions in 20 mg blank-hair to produce the CS equivalent to 10, 25, 50, 100, 250, 500 and 1000 pg/mg. QC samples were also prepared in blank-hair at concentrations of 10, 40, 200 and 750 pg/mg. Blank human hair samples were obtained from non-drug consuming children. CS and QCs solutions were stored at −20 °C.

Hair sample preparation

Hair was twice decontaminated using dichloromethane (immersions for 2 min) and once washed using warm water (immersion for 2 min). Hair was segmented and grinded into a fine and homogeneous powder using a ball mill (MM200, FicherScientific, Illkrich, France). Each 20 mg were incubated 10 min in 1 mL of phosphate buffer at pH 5.0 at 95 °C, in the presence of 2 ng of MDMA-d5, used as IS and appropriate volume of CS or QC working solutions when necessary. Liquid—liquid extraction was performed by 4 mL of a mixture of hexane/ethyl acetate (v/v: 1/1) after addition of 2 mL of carbonate buffer at pH 9.7. After fifteen minutes of agitation and five minutes of centrifugation, the organic phase was conserved and then evaporated to dryness. The residue was reconstituted in 80 μL of mobile phase and 10 μL were injected in the chromatographic system.

Method validation procedure

The method was validated for selectivity, linearity, detection and quantitation limits, accuracy, precision (repeatability and intermediate precision), specificity (matrix effect) and recovery according to the US Food and Drug Administration guidelines.

Selectivity, carry over

To investigate whether endogenous matrix constituents interfered with the assay, six drug-free matrix blank samples, zero samples and samples spiked at the LLOQ were analyzed according to the described procedure. Assay selectivity was defined by evidence of non-interference at retention times and ion channels identical to that of diphenidine and IS in the blank samples. A blank sample was also analyzed immediately following the highest CS in each run to monitor the carry over of diphenidine and IS.

Linearity

Calibration curves included a blank sample, a zero sample, and nine CS over the following concentration range: 10 pg/mg (lower limit of quantification [LLOQ]) to 1000 pg/mg (upper limit of quantification [ULOQ]). Six calibration curves obtained over a six-day period were taken into account for the study of linearity. Quantification was achieved by plotting the peak area ratios of diphenidine to the IS versus concentration followed by linear regression analysis, which was the best fitting model as determined by bias analysis. Back-calculated concentrations of the CS had to be within 85—115% of the nominal concentrations.

Lower limit of detection (LLOD) and quantification (LLOQ)

The LLOQ was defined as the lowest concentration for which an accuracy between 80 and 120% and a precision with a coefficient of variation of ± 20% or less that was obtained over six measurement. The LLOD is the lowest concentration of the compound that can be detected with a signal-to-noise ratio greater than 3:1 for the two transitions.

Accuracy and precision

Accuracy (measured value/nominal value) and precision (coefficient of variation) were determined for the four QC levels. For the intraday assay, six replicates of each QC level were processed the same day. For the interday assay,

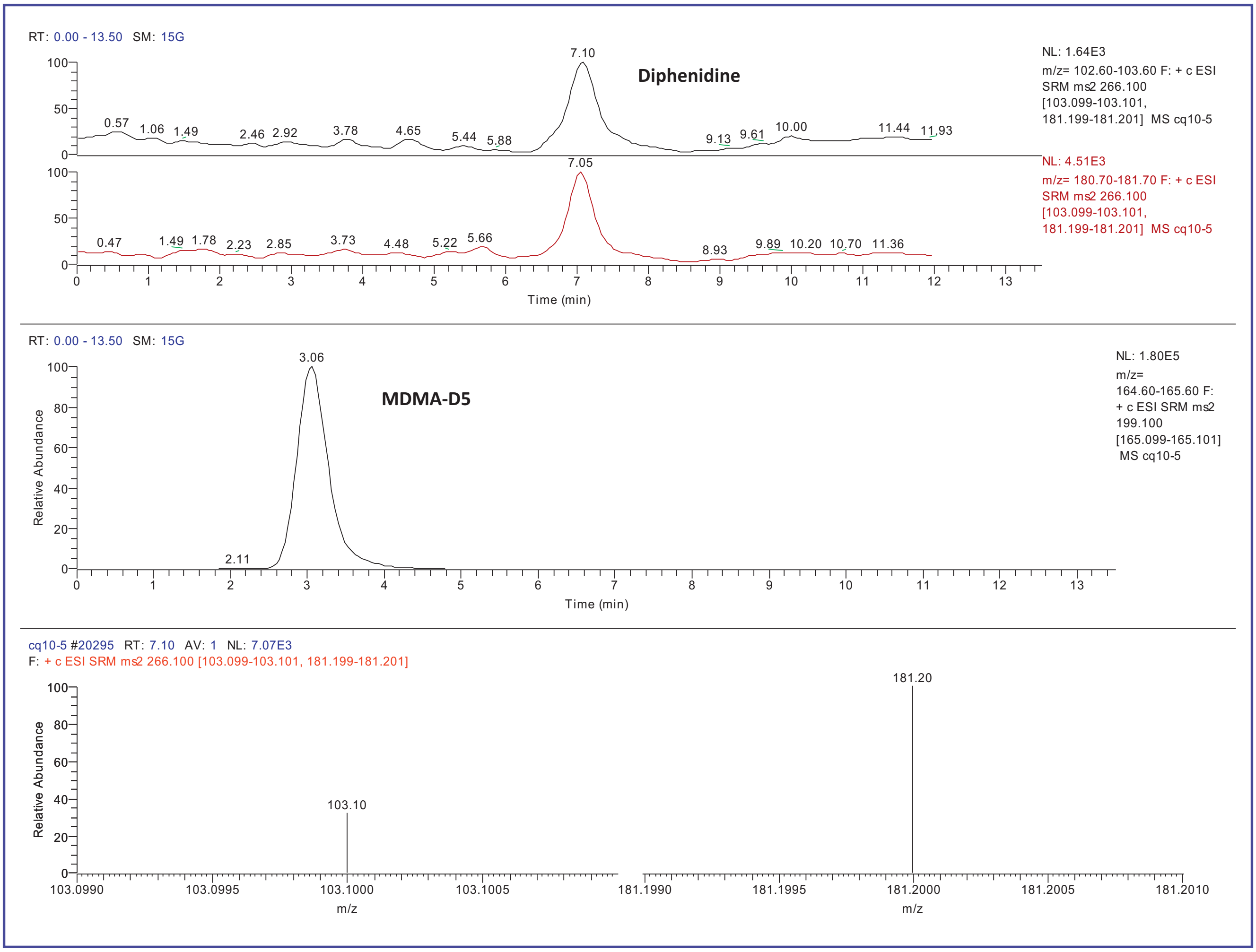

Figure 2. Chromatograms of the two specific transitions (m/z 266.1 → m/z 103.1 and m/z 181.2) of diphenidine and MDMA-D5 used as IS (m/z 199.1 → m/z 165.1) from blank-hair spiked with 10 pg/mg (LOQ).

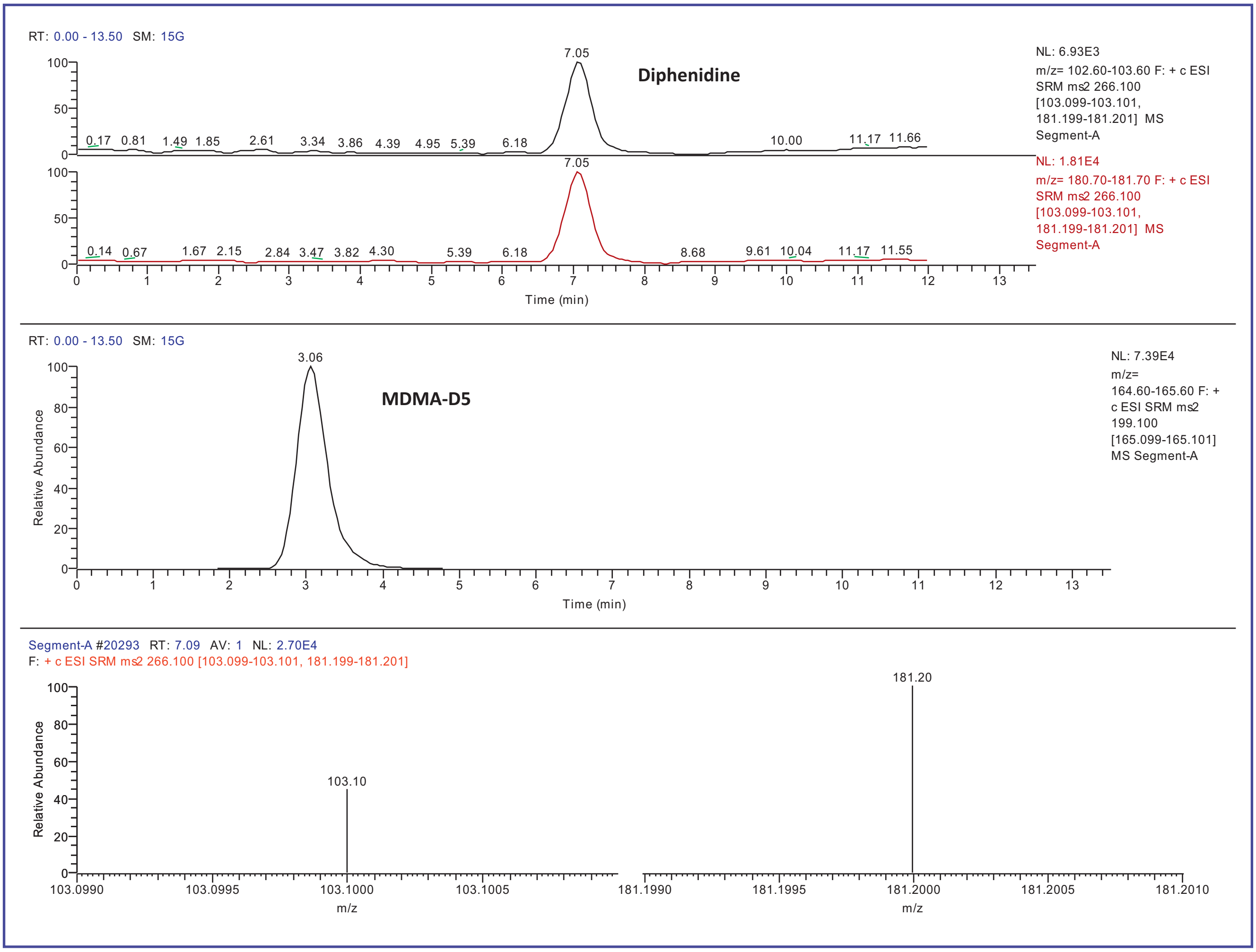

Figure 3. Chromatograms of the two specific transitions (m/z 266.1 → m/z 103.1 and m/z 181.2) of diphenidine and MDMA-D5 used as IS (m/z 199.1 → m/z 165.1) obtained from the first segment of the patient's hair (concentration = 123 pg/mg).

each QC level was processed six times three different days over a period of two weeks. The values obtained were analyzed using analysis of variance (ANOVA), which separated the intra-day and inter-day standard deviation and consequently the corresponding coefficients of variation (CV). An accuracy within the range 85—115% of the nominal values and a precision with a CV of ± 15% were required, except for the LLOQ for which a range of 80—120% and a CV of ± 20% were accepted for accuracy and precision, respectively.

Matrix effect and overall method recovery

Three procedures (A, B and C) were performed on pooled blank-hair at two concentration of 40 pg/mg and 750 pg/mg in order to evaluate matrix effect and overall method recovery:

(A) diphenidine was spiked in the mobile phase and directly injected;
(B) diphenidine was spiked afterwards in extracted blank matrix hair samples and injected;
(C) diphenidine was spiked in hair samples, the complete sample preparation procedure was carried through, and the samples were injected into the system.

The mean chromatographic peaks area obtained using the three procedures were compared. The ratios B/A, and C/A determined the matrix effect and the overall method recovery (excluding the hydrolysis phase) respectively, and were calculated in 6 experiments.

Results and discussion

LC—MS/MS analysis

Since diphenidine was structurally closed to amphetamine derivatives, and because no stable-isotope-labelled was available, MDMA-d5 was chosen as IS. Fig. 2 is the chromatogram obtained after extraction of spiked hair at the LLOQ (10 pg/mg). IS and diphenidine were separated with retention times of 3.1 and 7.1 min, respectively.

Method validation

Selectivity and carry over

No interferences from constituents of drug-free human hair at the retention times and the ion channels of both diphenidine and IS were observed. When a blank sample was

Table 2 Results of hair analysis in the reported case (pg/mg).

Compounds	Concentrations in hair				
	Segment 1 1 cm-20 mg	Segment 2 1 cm-20 mg	Segment 3 1 cm-20 mg	Segment 4 1 cm-20 mg	Segment 5 1 cm-20 mg
Drugs of abuse					
Diphenidine	123	79	89	ND	ND
Cocaine	850	471	752	2060	4658
Benzoylecgonine (BZE)	198	98	123	336	708
Ecgonine methyl ester (EME)	40	18	8	12	22
Anhydroecgonine methyl ester (AEME)	18	6	18	70	180
Cocaethylene	10	< 5	ND	ND	ND
Morphine	ND	ND	ND	5	12
Pharmaceuticals					
Aripiprazole	5392	3397	328	143	201
Tiapride	3775	5970	1154	44	15
Hydroxyzine	1910	966	578	210	238
Cetirizine	1017	373	192	36	55
Mianserine	307	312	88	63	76
Medazepam	479	291	146	66	87
Diazepam	312	136	106	80	100
Nordiazepam	592	351	180	90	107
Oxazepam	41	26	9	4	224
Temazepam	18	9	< 2	< 2	3.5
Levamisole	496	500	87	106	213
Tropatepine	353	101	102	10	7
Metoclopramide	170	370	117	12	< 10
Zolpidem	78	67	18	1	1
Zopiclone	56	78	31	2	ND
Lidocaine	< 5	14	17	37	85
Domperidone	< 5	< 5	5	14	37

ND: not detected (< 1—5 pg/mg of hair according to compounds).

analysed immediately after the highest calibration standard, mean carry over was lower than the limit of detection.

Linearity, limit of quantification and detection

The eight-point (including zero) calibration curve exhibited good linearity in the concentration range 10—1000 pg/mg with a correlation coefficient ranging from 0.9962 to 0.9992. Bias ranged from −9.6 to 11.6% for the back-calculated concentrations of the seven CS. The LLOQ was 10 pg/mg, demonstrating an intra-day and inter-day CV of 5.5 and 9.3%, respectively and an accuracy of 108.0%. The ULOQ was 1000 pg/mg. The LOD was defined as 2 pg/mg.

Precision and accuracy

Intraday and interday precision and accuracy of QC samples are summarized in Table 1. Intraday precision ranged from 5.5 to 7.6% and interday precision from 2.4 to 13.1% with accuracy between 101.6 and 108%.

Matrix effect and overall method recovery

The mean matrix effect was around 40% for both tested concentrations (Table 1). Unfortunately, no stable-isotope-labelled was available which could reduce this matrix effect. However, for the six-tested matrix, the CV was 8.8% for the low QC (40 pg/mg), and 4.3% for the high QC (750 pg/mg) showing that the matrix effect was reproducible. The mean overall recovery of diphenidine was 83.7 and 103.2% for the low and high QC ($n = 6$ for both QC), respectively. For the IS, no matrix effect was observed and overall recovery was 80.6% ($n = 12$). In this method, as many others compounds are also extracted, the buffer used for incubation of hair was set at pH 5.0, which allow extraction of many compounds with the minimum of degradation.

Case report

Diphenidine was only found in the first three proximal segments. Fig. 3 is the chromatogram obtained after extraction of the first segment of hair showing the usefulness of the method in real hair. Concentrations were 123 pg/mg in the first segment, closer to the scalp, and then 79 and 89 pg/mg in the two other segments. The sampling was realized 49 days after ingestion. According to the growth of hair, around 1 cm by month, diphenidine should have been found in the second segment if only one administration is accepted. However, since growth of hair is variable between 0.7 and 1.4 cm by month, the compound has been found in the three first segments. Besides, it is possible that some hair slipped in the stand, leading to a contamination of adjacent segment. Moreover, external contamination could have occurred with drug incorporation into the hair from sweat for example. This has been already described for methadone [11] or morphine [12] for example. So our results did not exclude a single administration of diphenidine as claimed by the patient. The only result of diphenidine in hair found in literature has shown concentration of 4400 pg/mg without any information about the consumption of the patient [10]. The metabolites of diphenidine were not measured in our patient even if it has been already shown that they could be present in hair [13].

Many other compounds were found in the hair (Table 2), all these compound measurements being validated in hair in our laboratory: cocaine (concentration decreasing from 4658 to 850 pg/mg from the extremity to the root of the strand, in accordance with decreasing consumption over the last 4—5 month) and its metabolites (benzoylecgonine, ecgonine methyl ester, cocaethylene), but also many pharmaceuticals such as aripripazole, tiapride, hydroxyzine and its metabolite cetirizine, mianserine, diazepam and its metabolites (nordiazepam, oxazepam and temazepam), tropatepine, zolpidem, zopiclone, levamisole and lidocaine (both probably contained in cocaine powders as cutting agents), in accordance with this patient's polyconsumption of psychoactive drugs that he claimed. Methoxetamine was not searched in our hair screening at the time of this analysis. These results are in accordance with the Helander et al. [7] results since the 14 non-fatal cases found in their study were always poly-drug users.

In our case, symptoms were consistent with a dissociative effect, including hallucination, amnesia and marked temporal disorientation. The patient reported that these effects were similar but stronger than those previously experienced as a chronic methoxetamine user; as he could not remember the amount he ingested, we can only suppose that the dose was around 1 g. The only stimulant effects observed were tachycardia and agitation, which occurred 11 hours after ingestion. Moreover, these effects may also have been due to the patient's anxiety rather than a specific pharmacological effect.

Conclusion

Diphenidine is a new dissociative designer drug acting as an NMDA antagonist, with a different chemical structure from that of arylcyclohexyamines such as ketamine or methoxetamine. Strong dissociative effects and amnesia were observed in a patient after his first exposure to this drug. The method for hair analysis described here allows the retrospective diagnosis of such poisoning cases. Diphenidine should be added to the list of drugs to be included in the toxicological screening of patients with psychodysleptic effects from recreational drug use [14].

Disclosure of interest

The authors declare that they have no competing interest.

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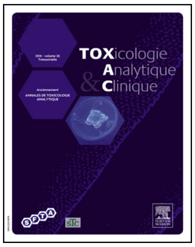

ORIGINAL ARTICLE

Retrospective identification of 25I-NBOMe metabolites in an intoxication case

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Received 15 November 2016; received in revised form 5 January 2017; accepted 5 January 2017
Available online 7 February 2017

KEYWORDS
Intoxication;
25I-NBOMe;
Metabolites;
High-resolution mass spectrometry;
Human liver microsomes

Summary New psychoactive substances (NPS) recently appeared on the recreational drugs market. Among them, N-Benzyl-Oxy-Methyl (NBOMe) derivatives are concern by mainly several reports in the literature of use and associated acute toxicity over the last 6 years, and the majority of the cases are related to 25I-NBOMe. We report a case of severe intoxication with a liquid mixture of NBOMes consumed nasally that has been retrospectively documented by 25I-NBOMe and metabolites determinations. A 29-year-old man experienced acute unconsciousness within one hour after a drop instillation in the nose of pink liquid. Initial examination on admission evidenced hypertonia and tremors, then partial seizure with secondary generalization, bilateral and reactive mydriasis, tachycardia, hypertension, hyperthermia and profuse sweating, suggestive of serotonin syndrome. He presented persistent cognitive and psychiatric abnormalities at M8. Liquid chromatography with high-resolution mass detection (LC-HRMS) revealed presence of

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http://dx.doi.org/10.1016/j.toxac.2017.01.001
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(i) three NBOMes in the pink liquid with 25I-NBOMe as main component, (ii) 25I-NBOMe in serum sampled at H3 (0.9 μg/L determined using Liquid chromatography with tandem mass spectrometry [LC-MS/MS]) but not in serum sampled at H15, and (iii) seven 25I-NBOMe metabolites in 2 urine samples (H3 and H15): two desmethyl-25I-NBOMes, a desmethyl-hydroxy-25I-NBOMe, a hydroxy-25I-NBOMe, a di-desmethyl-25I-NBome, a desmethyl-25I-NBOMe glucuronide and a hydroxy-25I-NBOMe glucuronide. These metabolites were retrospectively identified following *in vitro* investigations of NPS metabolism using analysis of samples collected after 25I-NBOMe incubation with a pool of human liver microsomes (HLMs) and cross-checking to *in silico* predicted biotransformations of the acquired data. The final goal of this analytical strategy is to record all the identified metabolites MS spectra in our HRMS and MS-MS libraries in order to performed routinely efficient detection of NPS users.

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Introduction

New psychoactive substances (NPS), sometimes designed as ''Research Chemicals'' or ''legal highs'' by users, concern substances that replicate chemical structures and/or pharmacological effects of classical drugs (ecstasy, amphetamine, cocaine or cannabis). These substances are easily available on the Internet on specialized website and their use has rapidly increased as well as their online availability. For instance, 98 new substances were detected in 2015 for the first time in the EU, bringing the number of new substances monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) to more than 560, of which 380 (70%) were detected in the last 5 years [1,2]. NPS are still poorly evaluated in terms of pharmacology or toxicology and the various galenic forms of NPS may be administered through routes. Among NPS, N-Benzyl-Oxy-Methyl (NBOMe) derivatives are mainly offered (sometimes under the name of ''mescaline'' or ''LSD'') as liquids, powders or small blotters soaked and were usually ingested orally/sublingually and, less commonly, nasally, through injection, vaginally, rectally, and smoked [3]. NBOMes are N-2-methoxybenzyl analogues of the respective 2C-X substituted phenethylamines derivatives (Fig. 1) exhibiting psychedelic properties, and were mentioned in PIHKAL (Phenethylamines I Have Known And Loved), by Alexander and Ann de Shulgin [4]. Pharmacological data available about 25I-NBOMe show a very potent 5-HT 2A receptor agonist action (Ki 0.044—0.15 nm). As a consequence, the typical user doses are reported to be in the sub-to-low milligram range. This is lower oral dosage than other NPS such as mephedrone and similar to other potent hallucinogens such as lysergic acid diethylamide (LSD) [5]. There are several reports in the literature of NBOMes use and associated acute toxicity from many countries in Europe, the United States and elsewhere over the last 6 years, and the majority of the cases are related to 25I-NBOMe: reported features include agitation/aggression, seizures, hyperthermia, tachycardia, hypertension and acute kidney injury [6—18]. In addition, 25 cases of acute toxicity related to the use of 25I-NBOMe were reported to the EMCDDA [5]. Besides, all of the published fatalities where an NBOMe has been detected are related to 25I-NBOMe rather than other NBOMe drugs [19]. All in all, 25I-NBOMe exhibits a non-negligible toxicity but only few reports included analytical confirmation of the use of 25I-NBOMe.

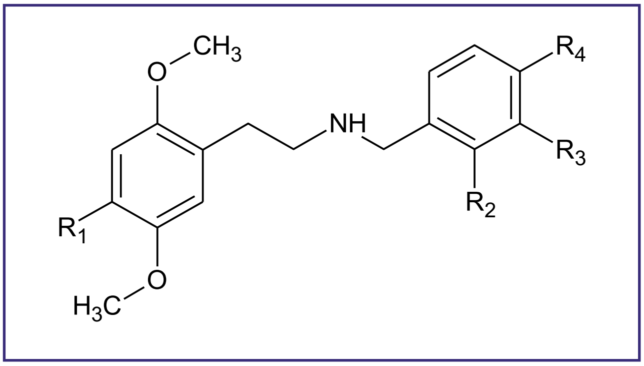

Figure 1. General structure of NBOMe derivatives.

Few months ago, we described a case of severe intoxication with 25I-NBOMe [20]. Within the following months, we subsequently characterized possible CYP- and UGT-dependent metabolites of NBOMe drugs (including 25I-NBOMe ones) by means of analyses of human liver microsome (HLM) incubates using high-resolution mass detection (LC-HRMS) in the way of recording all the obtained metabolite MS spectra in our library [21]. Indeed, this *in vitro—in silico* strategy should enable us to characterize relevant metabolites of 25I-NBOMe (and subsequently, to improve the detection window in biological matrices) and allow us to detect more efficiently 25I-NBOMe users (*in vivo*) [22,23]. Thus, in this case and using the incremented NPS screening mass spectra library, we could re-analyze the samples' HRMS data recorded few months before.

The aim of this work is to report this case of severe intoxication with a liquid mixture of NBOMes consumed nasally that has been retrospectively documented by 25I-NBOMe metabolites determinations, in the goal of illustrating an *in vitro—in silico—in vivo* strategy to take up the NPS analytical challenges.

Case report

The medical history of the 29-year-old patient includes generalized anxiety disorder, a single episode of psychotic decompensating after LSD use, alcohol consumption (occasional use), drug addiction with cannabis (occasional use) and LSD (weaned) and treatment by sertraline (with poor compliance). This young man experienced acute unconsciousness within one hour after a drop instillation in the nose of pink liquid.

Initial examination on admission (H1) evidenced hypertonia and tremors, then partial seizure with secondary generalization, bilateral and reactive mydriasis, tachycardia (120/min), hypertension (225/70 mmHg), hyperthermia (39 °C) and profuse sweating, suggestive of serotonin syndrome. Improvement was observed after diazepam (10 mg) and clonazepam (1 mg), allowing his transfer to Intensive Care Unit (ICU). In ICU, he presented neurological worsening and quiet coma interspersed with tonic movements (nearly opisthotonos) requiring sedation and aspiration pneumonitis. He received invasive ventilation during 24 hours and was discharge from ICU on day 11 with persisting confusion and memory disorder. During hospitalization, abnormalities in biological findings included: white blood cell 20.7 G/L, creatinine 142 μmol/L and urea 6.5 mmol/L (peak on day 1), lactate 7.9 mmol/L (peak on day 1), CK 25,800 U/L (peak on day 3), AST 891 U/L and ALT 853 U/L (peak on day 3). Chest X ray revealed a right pneumonitis. No epileptiform discharge was observed on EEG but electroencephalographic features suggest of a lowered seizure threshold (day 2 and day 4; not found on following EEG). The brain CT scan was normal and the brain MRI shown a symmetrical and bilateral impairment of the basal ganglia (day 5 and 12) and a centro-pontine lesion suggestive of a central pontine-extrapontine myelinolysis (day 12). Long-term evolution of brain MRI consisted in partial improvement with persisting abnormalities in caudate nucleus (day 62). The patient presented persistent cognitive and psychiatric abnormalities, with temporospatial disorientation, memory impairment and dysexecutive syndrome, requiring antipsychotic treatment (follow-up at day 240).

First-line toxicological analytical investigations were performed in urine and blood samples at H3: positive results consisted in blood and urine presence of benzodiazepine (using liquid chromatography with diode array detection) and a suspect low positive result for LSD (Enzyme Multiplied Immunoassay Technique). Several samples were subsequently sanded for second-line investigations: blood and urine sampled at H3 and H15, and a sample of the pink liquid nasally consumed by the patient.

Material and methods

Chemicals

β-OH-ethyltheophyllin (internal standard), methyl-clonazepam (internal standard), ammonium formate, formic acid, sulfosalicylic acid dihydrate, β-glucuronidase (*Helix pomatia*), alamethicin (*Trichoderma viride*), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate (G6P), 5-sulfosalicylic acid, were all purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Tetra-sodium salt of a reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany). A pool of human liver microsomes (HLMs) at a concentration of 20 mg of microsomal protein/mL, prepared from livers of 36 donors, including 11 women and 25 men, aged from 31 to 80 years, was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and preserved at −80 °C until use. 25I-NBOMe was provided by Cayman Chemical (Ann Arbor, USA). LC-MS grade water and acetonitrile were purchased from Biosolve (Dieuze, France), LC-MS grade methanol was provided by VWR (Fontenay-sous-Bois, France) and all other chemicals were of analytical grade and obtained from common commercial sources.

Liquid chromatography with high-resolution mass detection (LC-HRMS)

Sample preparation

Once thawed, HLMs (2 mg protein/mL for a final volume of 100 μL) were pre-activated by alamethicin on ice in an intermediate volume of 50 μL in 0.1 M Tris-HCl-$MgCl_2$ (10 mM $MgCl_2$ and 100 mM Tris-HCl solution) at pH 7.4. This mixture was added to a 50 μL-dried residue of three different concentrations of a 25I-NBOMe methanolic solution (0.4, 2.3 and 11.6 mM). Fifty μL of a cofactor mixture (5 mM UDPGA, 1.3 mM NADPH, 3.3 mM G6P and 0.5 U/mL G6PD) in 0.1 M Tris-HCl was then added. The enzymatic reaction was performed at 37 °C for 60 min and stopped by the addition of 100 μL of methanol-containing methyl-clonazepam at 1.25 mg/L and β-OH-ethyltheophyllin at 16 mg/L, used as internal standards for the chromatographic analysis. Samples were centrifuged at 4 °C for 14 min at 32,000 g and supernatants were diluted in 400 μL of 3% 5-sulfosalicylic acid before injection of 75 μL into the chromatographic system. One half of the urine samples were submitted to a prior beta-glucuronidase cleavage (20 μL of β-glucuronidase were added to 100 μL of urine and put at 37 °C for 30 min), and the unknown pink liquid was diluted (1:100) in methanol.

Method

Screening for NPS (including NBOMes and metabolites) was performed in biological samples (blood and urine) and in the unknown pink liquid using a previously reported LC-HRMS method [22,24]. The liquid chromatography system consisted of two binary solvent manager LC pumps, a sample manager autosampler and a column manager oven Acquity (Waters, Manchester, UK). Mass spectrometry data were acquired using a XEVO G2-XS QTOF (Waters, Manchester, UK) instrument controlled with MassLynx 4.1 software. After addition of internal standards (methyl-clonazepam and β-OH-ethyltheophyllin), extractions of samples (75 μL) were performed using an OASIS HLB on-line column (30 × 2.1 mm, 20 μm) (Waters, Manchester, UK). The chromatographic separation was performed using an ACQUITY HSS C18 column (150 × 2 mm, 1.8 μm, Waters) in an oven at (temperature

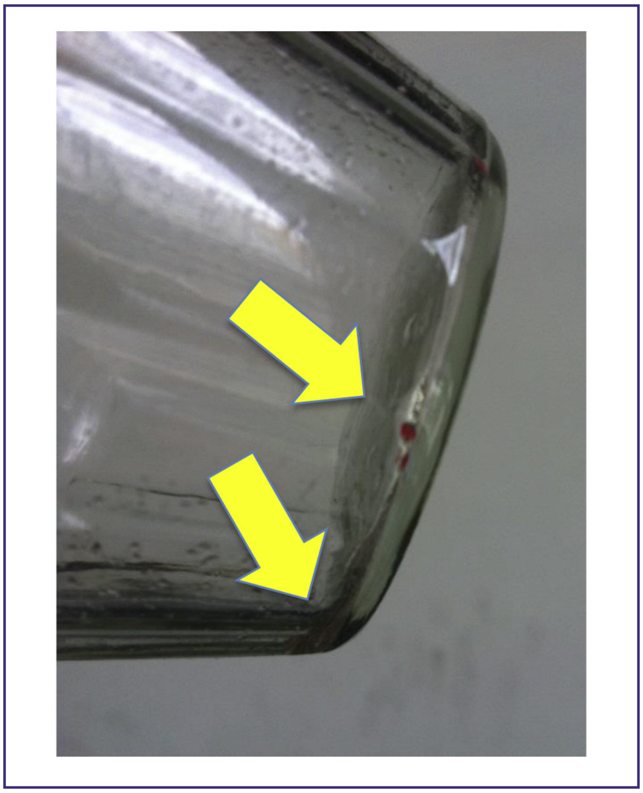

Figure 2. Pink liquid sample collected in a mustard jar.

of 50 °C), and mobile phases including ammonium formate buffer 5 mM, pH 3 (A) and acetonitrile in 1% formic acid (B); flow rate of 0.4 mL/min was used. For detection, mass spectrometric conditions were as follows: positive electrospray ionization interface (ESI+), ion spray voltage set at 20 V, source temperature set at 140 °C and desolvation temperature at 500 °C with a desolvation gas flow rate of 900 L/h, nitrogen as desolvation gas and argon as collision gas. Conditions for the time of flight mass spectrometer scan mode were as follows: scan range 100—1000 m/z for the function 1 and 50—1000 m/z, with a collision energy ramp from 10—40 eV, for the function 2. Data process was performed using ChromaLynx, TargetLynx, MassFragment and MetaboLynx associated software (Waters, Manchester, UK) using a homemade database of more than 1,400 substances including nearly 300 NPS (including 25I-NBOMe) or metabolites.

The obtained data were re-processed several months after using an incremented library including several 25I-NBOMe's metabolites identified beforehand as previously reported [21]. Indeed, the most relevant potential CYP- and UGT-dependent metabolites of 25I-NBOMes were investigated *in vitro* using HLM incubation and, subsequently, LC-HRMS analysis, using a previously published method [22,24]. Consecutively, the LC-HRMS acquired data are cross-checked to *in silico* predicted biotransformations using a software algorithm (MetaboLynx^{TM}). Briefly, sets of theoretically possible biotransformations of the studied drug (25I-NBOMe) were created based on the structure of the drug, including possible, hydroxylation, O- or N-dealkylation, deamination and glucuronidation transformations. The automated metabolite profiling process was completed through the use of the fragmentation interpretation software tool MassFragmentTM 4.1 (Waters), to enable software driven assignment of metabolite structures from fragmentation patterns.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS)

25I-NBOMe concentrations were subsequently determined in blood and urine samples using a LC-MS/MS method (XEVO TQS system, Waters, Manchester, UK). Briefly, 100 μL of blood or urine sample were deproteneinized (300 μL methanol) and 100 μL of supernatant were added with internal standard (100 μL of methanol-containing β-OH-ethyltheophyllin at 1.6 μg/L), subsequently diluted (200 μL of ammonium formate/0.1% formic acid buffer), and analyzed after separation by an AcquityTM UPLC HSS C18 column (1.8 μm, 2.1 × 150 mm, Waters, Manchester, UK) using a gradient of (A) 5 mM ammonium formate/0.1% formic acid buffer, and (B) ACN/0.1% formic acid as mobile phase at a flow-rate of 0.4 mL/min during 15 min The oven temperature was set at 50 °C and the injection volume was 10 μL. The gradient elution started with 87% of solution A for 0.5 min, decreased to 50% at 10 min, and to 5% at 10.25 min. The washing step with 95% of solution B was hold from 10.25 min to 12.5 min and the initial condition was applied from 12.5 min to 15 min. XEVO TQ-S tandem mass spectrometer was used for detection after positive electrospray ionization mode in the MRM mode using the following transitions: m/z 428.1 to 121.1 (for quantitation) and m/z 428.1 to 91.1 for 25I-NBOMe; m/z 225.1 to 124.1 (for quantitation) and 225.1 to 81.1 for IS (β-OH-ethyltheophyllin). The validation procedure of this assay complied with both the French Analytical Toxicology Society (SFTA) and international recommendations for the validation of new analytical methods [25,26] including linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and matrix effects. Calibration curves, estimated using 1/x weighted quadratic regression, were considered acceptable if the coefficient of determination (r^2) was at least 0.99. The LOD was defined as the lowest concentration with the two transitions presence and a signal-to-noise ratio of at least 3. The LOQ was the lowest concentration with the two transitions presence and an intra-assay precision CV% and a relative bias lower than 25%.

Results and discussion

Among NPS, the NBOMe derivatives are clearly used for their hallucinogenic properties. Close to their ''2C'' phenethylamine analogues, the NBOMes differ by the addition on the nitrogen (N) of a benzyl (B) methoxy (abbreviated OMe) radical that provided a greater affinity for the 5-HT2A serotonin receptors. The variety of NBOMe derivatives comes from the nature of the R_1 substituent (Fig. 1): in our case, iodine for 25I-NBOMe, hydrogen for 25H-NBOMe and chlorine for 25C-NBOMe. There are no animal/human studies on the toxicological properties of these potent serotonergic agonists. Data on their clinical effects rely exclusively on case-reports of human poisoning. Their toxicological pattern combines a sympathomimetic toxidrome possibly accompanied by serotonergic signs. The alleged effective doses are low, typically

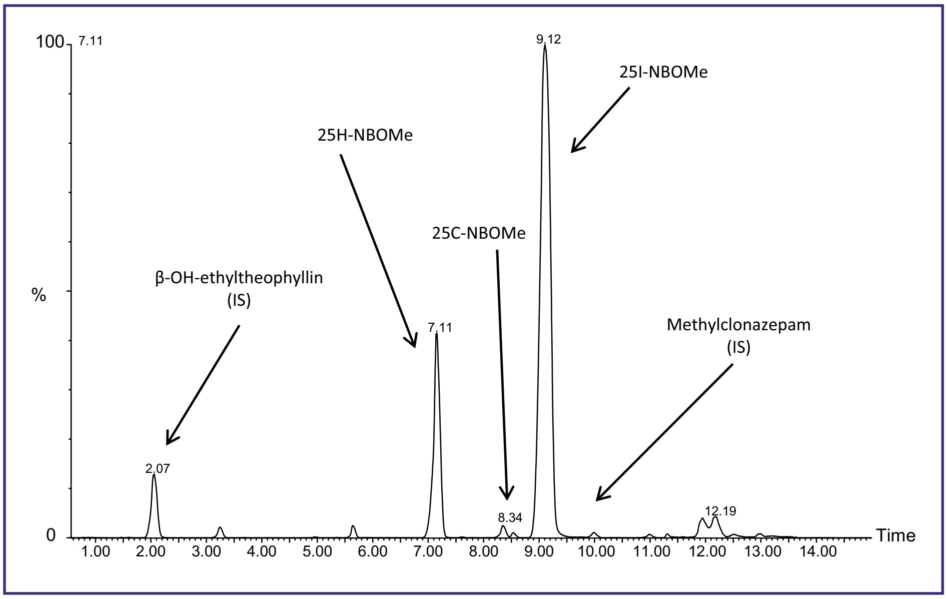

Figure 3. HRMS chromatogram of the pink liquid.

about 500 micrograms [5]. In this case, according to the severity of the clinical picture despite an assumed low dose (not evaluated), this case reminds us how acute poisoning with NBOMe derivatives may be dangerous and carry the risk of long-term sequelae.

The pink liquid sample was collected in a mustard jar (Fig. 2): a few drops of pink liquid with a pleasant and fruity smell. LC-HRMS revealed presence of three NBOMes: 25I-NBOMe (theoretical m/z 428.0723, rt 9.11 min, main chromatographic pic), 25H-NBOMe (m/z 302.1756, rt 7.11 min) and 25C-NBOMe (m/z 336.1366, rt 8.34 min) (Figs. 3 and 4).

Indeed, the pink liquid instilled by the patient in his nose was not pure. Even though its main active component is 25I-NBOMe, it also contained 25H-NBOMe and 25C-NBOMe. This result is coherent with data found in the literature [8,15,27,28], i.e. 25H-NBOMe, which is always found as an impurity in 25B-NBOMe and 25I-NBOMe products. The reason for the 25H-NBOMe's presence could be that 25I-NBOMe is most commonly synthesized by reductive coupling of methoxybenzaldehyde with 2C-I. Within the synthesis of 2C-I is an iodination step of a precursor common to both 2C-I and 2C-H [29].

The positive results obtained in serum and urines samples are summarized in Table 1. It is of note that (i) neither 25H-NBOMe or related metabolites, nor 25C-NBOMe or related metabolites, was detected in the patient's biological samples, and that (ii) drug positive results included drugs of medical support and sertraline (patient's treatment) with concentrations in serum (H3) in therapeutic range.

We were able to detect 25I-NBOMe in serum sampled at H3 (and quantified using LC-MS/MS* at 0.9 μg/L) but not in serum sampled at H15 (*LOD: 0.2 μg/L, LLOQ: 0.5 μg/L, ULOQ: 100 μg/L, interday CV and bias (*n* = 5) < 20% in blood and in urine, coefficients of determination superior to 0.99 for each calibration curve).

This result is coherent with (i) extensive metabolism of NBOMes [13] and (ii) previous reported concentrations of 25I-NBOMe in blood that are in the 0.03 to 8 μg/L range. A 25I-NBOMe concentration of 7.5 μg/L was reported in the serum of a 18-year-old female following intoxication by 25I-NBOMe [8]. Following intoxication by 25I-NBOMe, a concentration of 0.76 μg/L was determined in the serum of a 18-year-old male admitted in emergency department [7]. In a case of a suicide attempt after LSD ingestion, which was analytically determined to be 25I-NBOMe instead, a serum concentration of 0.034 μg/L was reported [12]. In a traumatic fatality of a 19-year-old man who had ingested blotter paper containing 25I-NBOMe, postmortem 25I-NBOMe concentrations in peripheral blood, heart blood and urine were 0.40, 0.41 and 2.86 μg/L, respectively [11]. Nevertheless, the possibility of *in vitro* instability of 25I-NBOMe should be considered for blood concentration interpretation as Johnson et al. reported a loss of 50% in 25I-NBOMe blood concentration over a 7-day storage period at 4 °C [30].

In vitro experiments using HLM incubation and LC-HRMS analysis were performed in order to investigate the potential CYP- and UGT-dependent metabolites. Regarding the three concentrations of 25I-NBOMe tested for incubation experiments, a satisfactory metabolite production was observed after incubation of 25I-NBOMe at 2.3 mM of 25I-NBOMe. Accordingly, the following results are those observed for this concentration. Several metabolites of 25I-NBOMe were detected including:

- CYP products: three desmethyl-25I-NBOMes (theoretical m/z 414.0566, rt 6.8 min [M1], 6.86 min [M2] and 7.25 min [M3]), two di-desmethyl-25I-NBOMes (m/z 400.0404, rt 5.45 min [M4] and 6.02 min [M5]), two hydroxylated 25I-NBOMes (m/z 444.0666, rt 7.12 min [M6] and 7.58 min [M7]), a hydroxylated desmethyl-25I-NBOMe (m/z 430.0560, rt 6.22 min [M8]) and a N-dealkyl-25I-NBOMe, product of the loss of the methoxy—benzyl moiety (m/z 308.0142, rt 5.51 min [M9]),
- and UGT products: two 25I-NBOMe glucuronides (m/z 604.1064, rt 6.00 min [M10] and 6.36 min [M11]), two hydroxy-25I-NBOMe glucuronides (m/z 620.0993, rt

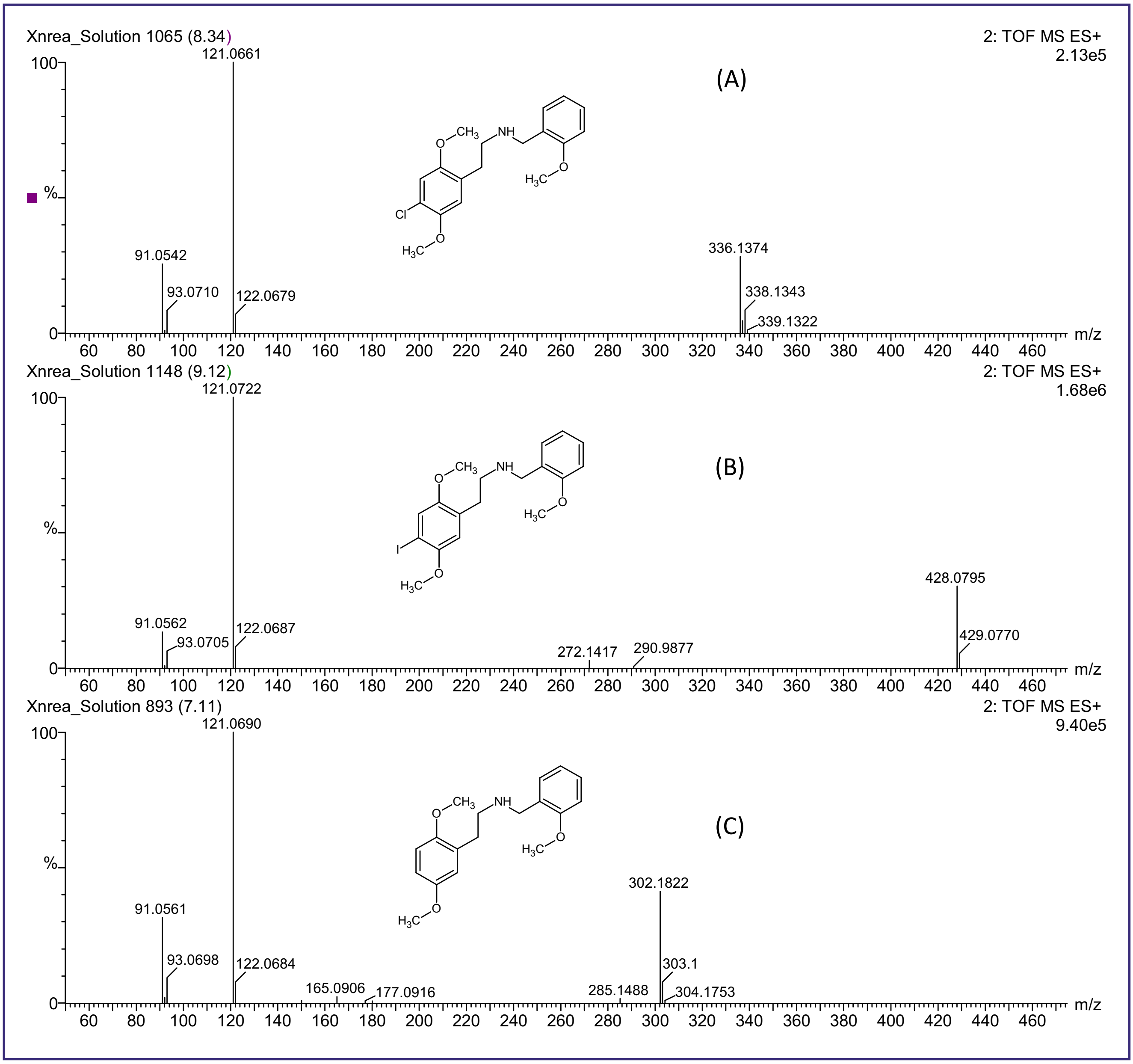

Figure 4. HRMS mass spectra of 25C-NBOMe (A), 25I-NBOMe (B) and 25H-NBOMe (C).

5.76 min [M12] and 5.95 min [M13]), three desmethyl-25I-NBOMe glucuronides (m/z 590.0887, rt 5.41 min [M14], 5.54 min [M15] and 6.09 min [M16]), and three di-desmethyl-25I-NBOMe glucuronides (m/z 576.0741, rt 4.31 min [M17], 4.88 min [M18] and 5.04 min [M19]) (Fig. 5) [21].

The *in vitro*-produced metabolites of 25I-NBOMe were not in conflict with those predicted by the Metabolynx^{TM} 4.1 software, as all the predicted metabolites were detected. Thus, the *in silico* predicted biotransformations contribution consists mainly to audit the *in vitro*-produced metabolites. These recorded 25I-NBOMe metabolites are similar to those recently reported in the literature. The biotransformation reactions include hydroxylation, O-demethylation, N-dealkylation, dehydrogenation, and combinations thereof. All methoxy groups can be demethylated and hydroxylation preferably occurs at the NBOMe ring. The main biotransformation seems to be O-demethylation, followed by O-di-demethylation and hydroxylation [31,32]. It is of note that the major CYP enzyme involved in the metabolism of 25I-NBOMe was identified as CYP3A4: as a consequence, users of 25I-NBOMe may be subject to drug—drug interactions if 25I-NBOMe is taken with a strong CYP3A4 inhibitor [33].

Seven of these 25I-NBOMe metabolites were retrospectively identified in the urine samples (H3 and H15): two desmethyl-25I-NBOMes (M1 and M2), a desmethyl-hydroxyl-25I-NBOMe (M8), a hydroxylated 25I-NBOMe (M6), a di-desmethyl-25I-NBome (M5), a desmethyl-25I-NBOMe

Table 1 Positive results obtained in serum and urine samples.

Component	Serum		Urine	
	H3	H15	H3	H15
Diazepam	196	Presence	Presence	Presence
Nordiazepam	3	Presence	Presence	Presence
Oxazepam	Nd	Nd	Presence	Presence
Sertraline	82	Presence	Presence	Presence
Clonazepam	12	Presence	Presence	Presence
25I-NBOMe	0.9	Nd	1.4	1.2
Desmethyl-25I-NBOMe (M1)	Nd	Nd	*3.36*[a]	*3.50*[a]
Desmethyl-25I-NBOMe (M2)	Nd	Nd	*0.54*[a]	*0.35*[a]
Desmethyl-hydroxy-25I-NBOMe (M8)	Nd	Nd	*0.30*[a]	*0.19*[a]
Hydroxy-25I-NBOMe (M6)	Nd	Nd	*0.34*[a]	*0.21*[a]
Di-desmethyl-25I-NBOMe (M5)	Nd	Nd	*0.81*[a]	*0.43*[a]
Desmethyl-25I-NBOMe glucuronide (M14)	Nd	Nd	*2.90*[a]	*3.08*[a]
Hydroxy-25I-NBOMe glucuronide (M13)	Nd	Nd	*0.16*[a]	*0.50*[a]

Concentrations in µg/L; Nd: not detected; presence: identification without quantification.

[a] Metabolic ratio using peak area: area of metabolite/area of parent drug (25I-NBOMe)

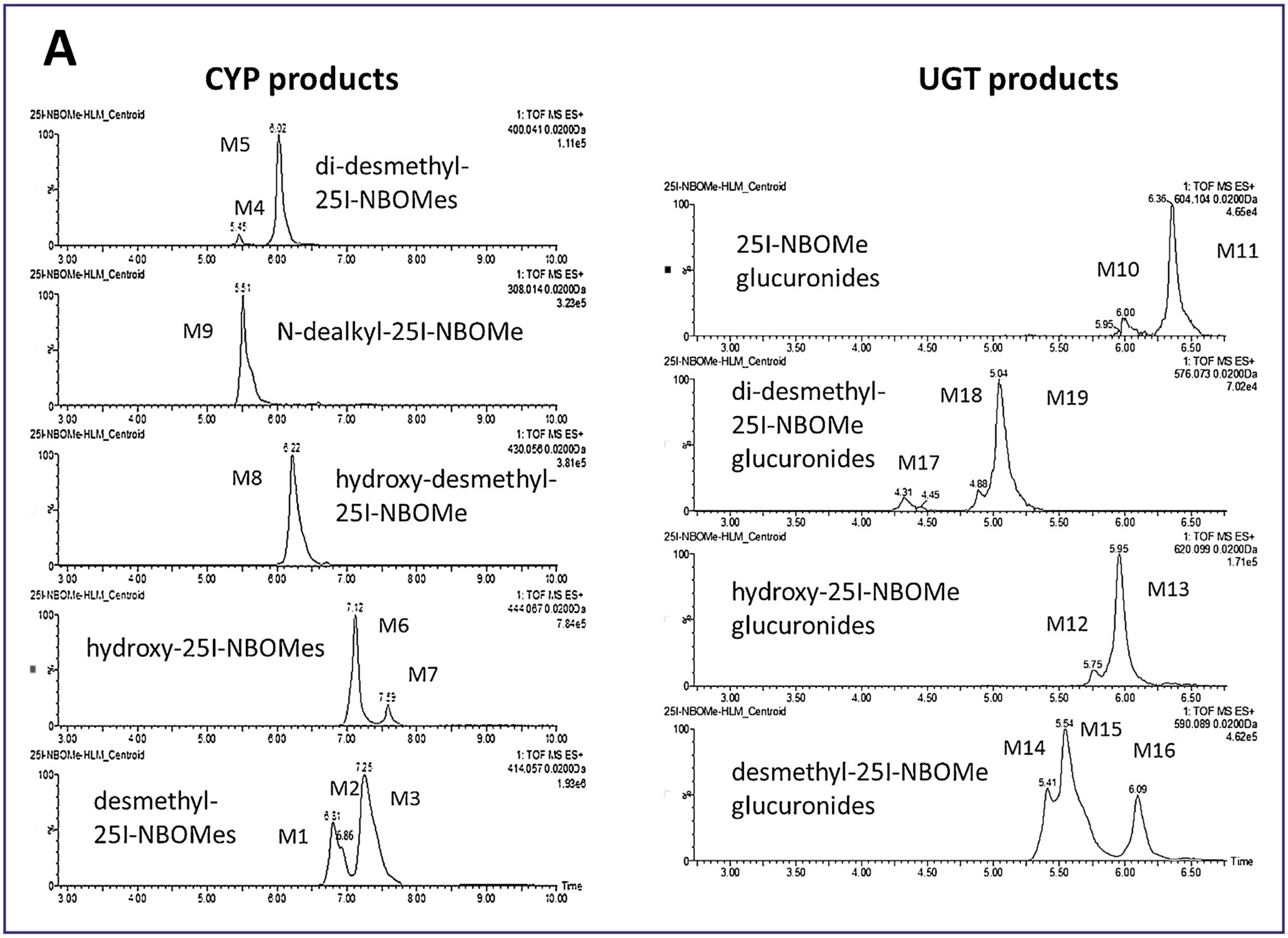

Figure 5. Metabolites of 25I-NBOMe produced by *in vitro* experiments using HLM: chromatograms (A) and HRMS spectra (B) (fragmentation patterns of M4, M10, M11, M12, M17 and M18 are not available owing to a too low *in vitro* production).

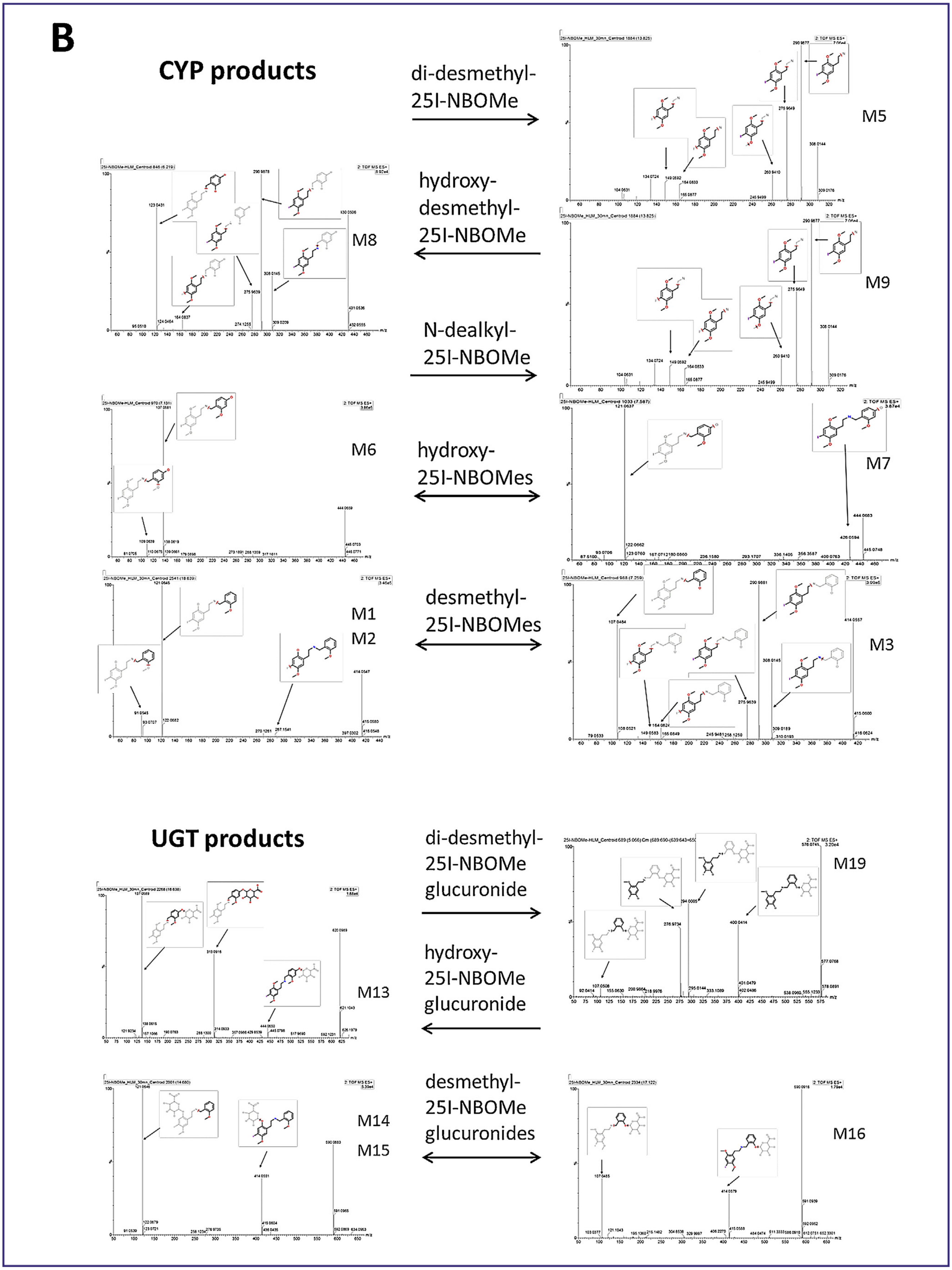

Figure 5. (*Continued*).

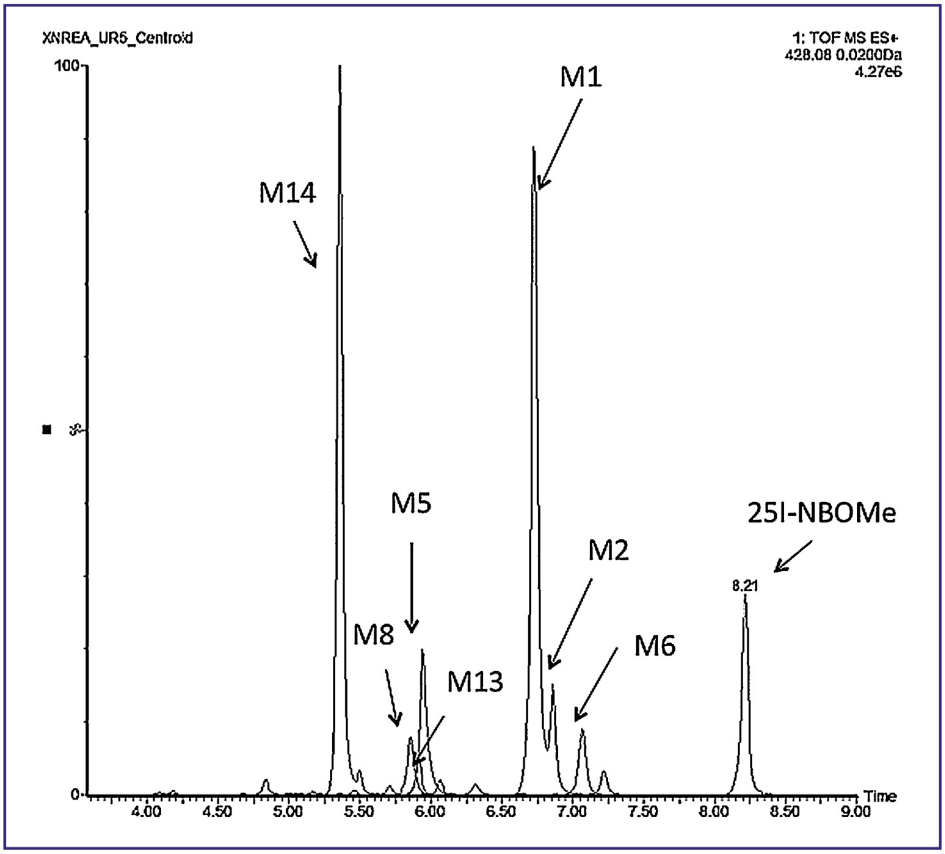

Figure 6. HRMS chromatogram of 25I-NBOMe metabolites in urine (H3).

glucuronide (M14) and a hydroxy-25I-NBOMe glucuronide (M13) (Table 1 and Fig. 6). Considering relative peak area surfaces, the desmethyl-25I-NBOMe (M1) was the most abundant metabolite in the two urine samples, followed by desmethyl-25I-NBOMe glucuronide (M14).

The identification of such metabolites is of importance in the challenge of NPS analytical detection. Indeed, trying to detect the parent drug could lead to false-negative results (i) when the delay between consumption and sampling has been too long and (ii) owing to very low concentration of the parent drug in blood and urine as observed with NBOMes. Therefore, identification of their metabolites could improve their detection window in biological matrices. Thus, in this intoxication case, some metabolites are clearly more abundant in urine than the parent drug (Fig. 6). Based on these case's data and a comparison with synthesized reference standards for potential metabolites, specific and abundant 25I-NBOMe urine targets seem to be desmethyl-25I-NBOMes and desmethyl-25I-NBOMe glucuronides. This result is close to preliminary findings of Wohlfarth et al. [32]. Nevertheless, owing to foreseeable interindividual variability in pharmacokinetic of 25I-NBOMe, additional *in vivo* data (from intoxication cases) are needed to confirm this statement and to determine relevant urinary metabolites of 25I-NBOMe. Indeed, results in only one human urine were reported by Caspar et al., and also by Wohlfart et al. [31,32]: all in all, there are only 2 cases of human urine data in the literature concerning 25I-NBOMe's metabolites.

Conclusion

NPS are constantly evolving — both in their nature and appearance — with pitfall related to a denomination sometime similar to that of known and less toxic product, a presentation in an unusual shape, administration through unusual routes, and severe and unexpected clinical findings. According to the severity of the clinical picture, despite an assumed low dose, this case reminds us how acute poisoning with NBOMes (active from sub-milligram doses) may be dangerous and carry the risk of long-term sequelae.

Regarding NPS characterization, we use a combination of *in vitro* (*in silico*) and *in vivo* investigations to take up the NPS analytical challenges using LC-HRMS analytical method. It is now obvious that high-resolution mass spectrometry offers higher mass precision, which greatly facilitates identification of unknown compounds, and apparently shows the best performance in comparative studies for drug screening assays [34,35]. *In vitro* investigations of NPS metabolism involve analysis of samples collected after drug incubation with a pool of human liver microsomes (HLMs) and acquired data are cross-checked to *in silico* predicted biotransformations. Secondarily, the final validation of identified NPS metabolites is performed by confrontation to real *in vivo* NPS cases (urine or blood assays) results [36]: this *in vivo* validation can be performed several months after the intoxication, as HRMS allows subsequent re-analysis of the samples' HRMS data previously recorded, as completed in this 25I-NBOMe case. The final goal of our *in vitro* (*in silico*) strategy is to record all the identified metabolites MS spectra in our HRMS and MS-MS libraries in order to performed routinely efficient (*in vivo*) detection of NPS users.

Disclosure of interest

The authors declare that they have no competing interest.

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Toxicologie Analytique & Clinique (2017) **29**, 82—89

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ORIGINAL ARTICLE

2,5-dimethoxy-4-chloroamphetamine, a LSD-like designer drug: Clinical and analytical documentation of non-fatal exposure in five patients

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Received 17 November 2016; received in revised form 6 January 2017; accepted 6 January 2017
Available online 15 February 2017

KEYWORDS
2,5-dimethoxy-amphetamines;
DOC;
LSD-like designer drugs

Summary Designer drugs appear to be increasing in popularity because of easy obtaining on the Internet, the lack of ability to identify the compound(s) in routine toxicology screening and being marketed as 'legal' substitute of drugs of abuse. 2,5-dimethoxy-4-chloroamphetamine (DOC) is a substituted alpha methylated phenethylamine and acts as a selective serotonin receptor partial agonist. There is limited literature on this particular compound, which is rarely encountered in biological matrices, and only one case-report attributed death to the use of this drug alone. We present five cases of non-fatal exposure to DOC with sever clinical symptoms (ages range 18—23 years). DOC was identified using data from LC/UV and GC/MS screening procedures. DOC in plasma and urine samples was quantified using a validated LC/MS/MS method and results showed that DOC plasma concentrations were less than 18 μg/L and DOC urine concentrations ranged from 300 to 1300 μg/L. This is the first documentation of DOC consumption in France, and in 4 cases, the patients believed that they were ingesting LSD. This indicates the emergence of designer drugs use as 'legal' LSD substitutes and we are concerned by the discrepancies in legal status of such compounds in the EU, which limit the effectiveness of drug enforcement policies.

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http://dx.doi.org/10.1016/j.toxac.2017.01.002
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Introduction

New Psychoactive Substances, often referred to as NPS family or designer drugs, are a range of different chemical compounds commonly sold through internet sites as legal substitutes for chemical drugs of abuse. In recent years, besides the classic designer drugs of the amphetamine-type, series of new drug classes appeared on the illicit drugs market such as 2,5-dimethoxy-amphetamines (DOs) or phenethylamines (2Cs) with different substitution at position 4, beta-keto-amphetamines and the phencyclidine derivatives [1]. The DOs and 2Cs designer drugs are not well characterized in human. There are some notable differences between traditional amphetamines and the newer alternatives. Despite the structural similarity of these synthetic derivatives, minor alteration may have significant impact of their pharmacodynamic profiles [2—5]. These drugs may be encountered illicitly in a variety of forms, including tablets, capsules, powders and LSD-like blotters (for 2Cs) or liquids (for DOs) [5].

The chemical structures of typical drugs of the class of 2,5-dimethoxy amphetamines are shown in Fig. 1. Most of these drugs were described by Shulgin [6], and as they have a high abuse potential, many were scheduled in most countries. Some information is available on their pharmacologic properties: action as agonists or antagonists at different 5-HT2 receptor subtypes. The harm associated of these series in not well understood, although it is clear that they are not without risk [5].

2,5-dimethoxy-4-chloroamphetamine (DOC) is a substituted alpha methylated phenethylamine and acts as a selective serotonin receptor partial agonist. Shulgin in his PIHKAL book classified DOC as an archetypical psychedelic, which he further defined as a certain drug that allows for increased personal insight and expansion of one's mental and emotional horizon [6]. There is limited literature on this particular compound, which is rarely encountered in biological matrices [7,8], and only one case-report attributed death to the use of this drug alone [9].

In this article, we present five cases of non-fatal exposure to DOC with sever clinical symptoms, in addition to the analytical procedures followed for its identification and quantification in biological matrices.

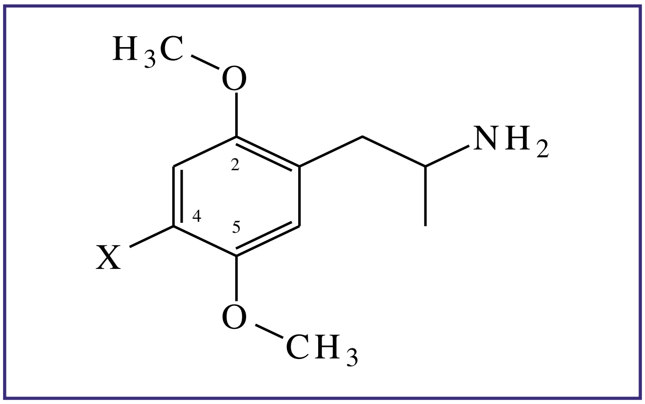

Figure 1. Chemical structures of 2,5-dimethoxyamphetamines (X = CH_3 for 2,5-dimethoxy-4-methylamphetamine [DOM]; C_2H_5 for 2,5-dimethoxy-4-ethylamphetamine [DOEt]; I for dimethoxy-4-iodoamphetamine [DOI]; Cl for 2,5-dimethoxy-4-chloroamphetamine [DOC]; Br for 2,5-dimethoxy-4-bromoamphetamine [DOB]).

Clinical cases

Case history 1

A 18-year-old man, with no known medical history, has been admitted to an intensive care unit (ICU) for seizures, agitation and hallucinations following the consumption of 1 LSD blotting paper and MDMA at a rave-party. The patient made incoherent speech, and presented mydriasis with blood pressure of 130/50 mmHg and a heart rate of 84 beats/min. The poisoning severity score (PSS) calculated at h6 after consumption, was 2. The patient was discharged from the ICU on the next day with good recovery. Blood and urine samples were obtained for toxicological analyses approximatively 6 hours after drug ingestion. Urinalysis drug test was very positive (> 2000 μg/L equivalent amphetamine and > 1000 μg/L equivalent MDMA) and blood ethanol was negative. No blotting paper or powder was obtained for analysis.

Case history 2

A 21-year-old man, with no known medical history, has been admitted to an intensive care unit (ICU) for agitation and hallucinations following the consumption of 1.5 LSD blotting paper according to the attendees at the same rave-party as for patient described in case 1. The patient presented tachycardia (135 beats/min) and bilateral mydriasis with blood pressure of 133/74 mmHg. The PSS calculated at h8 after consumption, was 2. The patient needed contention and Valium® administration. The patient was discharged from the ICU on the same day with good recovery. Blood and urine samples were obtained for toxicological analyses approximatively 8 hours after drug ingestion. Urinalysis drug test was negative and ethanol blood concentration was 0.13 g/L. No blotting paper was obtained for analysis.

Case history 3

A 23-year-old man, with no known medical history, has been admitted to an emergency department (ED) for agitation following the consumption of alcohol, ketamine, cocaine, cannabis and LSD during a rave-party. The patient presented visual hallucinations (at h24) with blood pressure of 120/80 mmHg and a heart rate of 93 beats/min. The PSS, calculated at h11 after consumption, was 2. The patient was discharged on the next day with good recovery. Blood and urine samples were obtained for toxicological analyses approximatively 11 hours after drug ingestion. Urinalysis drug test was very positive for cannabis and cocaine, and blood ethanol concentration was 1.28 g/L. No blotting paper or powder was obtained for analysis.

Case history 4

A 16-year-old man, known for drug abuse (cannabis and amphetamine), has been admitted to a Paediatric Emergency Department for near death experience and serious

Table 1 MS/MS condition for 7 compounds belonging to DO-series.

	SRM1	SRM2	Declustering potential (V)	Collision energy (V)
DOC	230.1 > 213.0	230.1 > 185.0	56	15 and 21
DOB	276.1 > 259.1	276.1 > 231.0	46	17 and 27
DOI	322.1 > 277.0	322.1 > 304.9	56	27 and 17
2,5-DMA	196.1 > 179.1	196.1 > 151.1	36	15 and 23
3,4-DMA	196.1 > 179.1	196.1 > 151.1	46	15 and 27
DOM	210.2 > 193.1	210.2 > 165.0	51	15 and 25
DOEt	224.2 > 207.1	224.2 > 179.2	41	17 and 25
MM	2611 > 244.1		61	15

anxiety following the consumption of 2 blotting papers that he identified as 'Trip', i.e. LSD, at a private event. The patient presented hallucinations and bilateral mydriasis with PSS = 2 calculated at h4 after consumption. The patient was discharged two days later with good recovery. Blood and urine samples were obtained for toxicological analyses approximatively 4 hours after drug ingestion. Urinalysis drug test was negative for amphetamine and positive for cannabis. Blood ethanol concentration was negative. No blotting paper or powder was obtained for analysis.

Case history 5

A 21-year-old man, with no known medical history, has been admitted to an intensive care unit (ICU) for seizures following the consumption of a drug designer bought on the Internet as a 'LSD derivative'. The patient presented hyperthermia (39.5 °C), tachycardia (150 beats/min) and mydriasis with blood pressure of 150/80 mmHg. The PSS, calculated at h5 after consumption, was 3. His management needed intubation and ventilation and he was discharged from the ICU three days later with good recovery. Blood and urine samples were obtained for toxicological analyses approximately 5 hours after drug ingestion. Urinalysis drug test was very positive for: amphetamine (but negative for MDMA), cannabis, opioids, and positive for methadone. Blood ethanol concentration was negative. Concomitantly to the admission of this patient, the National Gendarmerie demanded the analysis of a powder bag obtained at the patient's relative house. The bag contained a white powder and labelled as (DOC not for human consumption 250 mg). Upon reception in our laboratory, the weight of the bag content was 185 mg of powder.

Materials and methods

Routine toxicological screening was performed according to several methods described in details previously [10]: HPLC-UV/DAD based methods (Agilent, HP1100 or 1200, Les Ulis, France) for plasma, urine and powder samples after two distinguished extraction procedures: extraction of neutral and basic compounds under simple alkaline pH and extraction of basic compounds under alkaline pH followed by acidic back-extraction. Urine and powder samples were also purified using alkaline extraction by diethylether followed by derivatization with heptafluorobutyric anhydride (HFBA, Fluka-Sigma-Aldrich, Saint Quentin Fallavier, France) and analysed by GC/MS system (Agilent, HP6890/HP5973N, Les Ulis, France). The different solvents and reagents were HPLC grade or analytical grade.

Specific quantitative analysis of plasma and urine samples was realized by a two-dimension liquid chromatography-mass spectrometry system (AB-Sciex 4000 QTRAP, Les Ulis, France). The chromatographic system was a Shimadzu UFLC-XR equipped with two LC20 high-pressure elution pumps, one LC20 concentration pump and an oven maintained at 45 °C. A sample (100 μL), to which 20 μL IS methanolic solution (methylmilnacipran, 1 mg/L) is added, was treated by methanol (150 μL) to precipitate the proteins and then the supernatant was diluted four-fold with water. A volume of 10 μL was injected on an on-line extraction Strata®-X precolumn (25 μm, 20*2 mm, Phenomenex, Le Pecq, France) conditioned with 2 mM ammonium formate solvent (2 mL/min). The precolumn was connected after 1.0 min to the Kinetex® phenyl-hexyl column (100 *3 mm, Phenomenex). The mobile phase was a mixture of ammonium formate (2 mM + 0.2% formic acid) buffer and acetonitrile (containing also 2 mM ammonium formate and 0.2% formic acid). The gradient solvent (flow-rate 0.6 mL/min) was as followed: 90/10 (v/v) at time 0, for 1 min, to 65/35 at 9.5 min and 10/90 at 10 min for 1 min before return to initial conditions. Total run time was 12 min without equilibration time between runs.

The MS conditions were as followed: positive electrospray ionization mode (ESI+) with a source temperature at 500 °C and ion-spray voltage of 5.5 kV. Multiple Reaction Monitoring (MRM) was performed with two transitions per compound and one for the IS. Seven molecules were evaluated with this procedure: DOC, DOI, DOB, DOM, DOET, 2,5-DMA, 3,4-DMA (Table 1). Reference standards of all compounds were obtained as pure powder or as 1 mg/mL methanolic solutions from LGC Standards (Molsheim, France).

This method was validated using the validation consensus of the SFTA [11]. Linearity was assessed with a 7-point calibration curve (10, 20, 50, 100, 250, 500 and 1000 μg/L) by plotting ratio of peak area and the IS area versus nominal analytes concentrations. The best fit was obtained using linear least squares regression when possible. Otherwise, quadratic regression was applied. The limit of quantification was chosen as the first standard point of calibration curve. The intra-day and inter-day precision were evaluated by analysing 3 different spiked plasma control levels (15, 300 and 750 μg/L), 4 times per day and during 4 days. Results were expressed as the % relative standard deviation (% RSD).

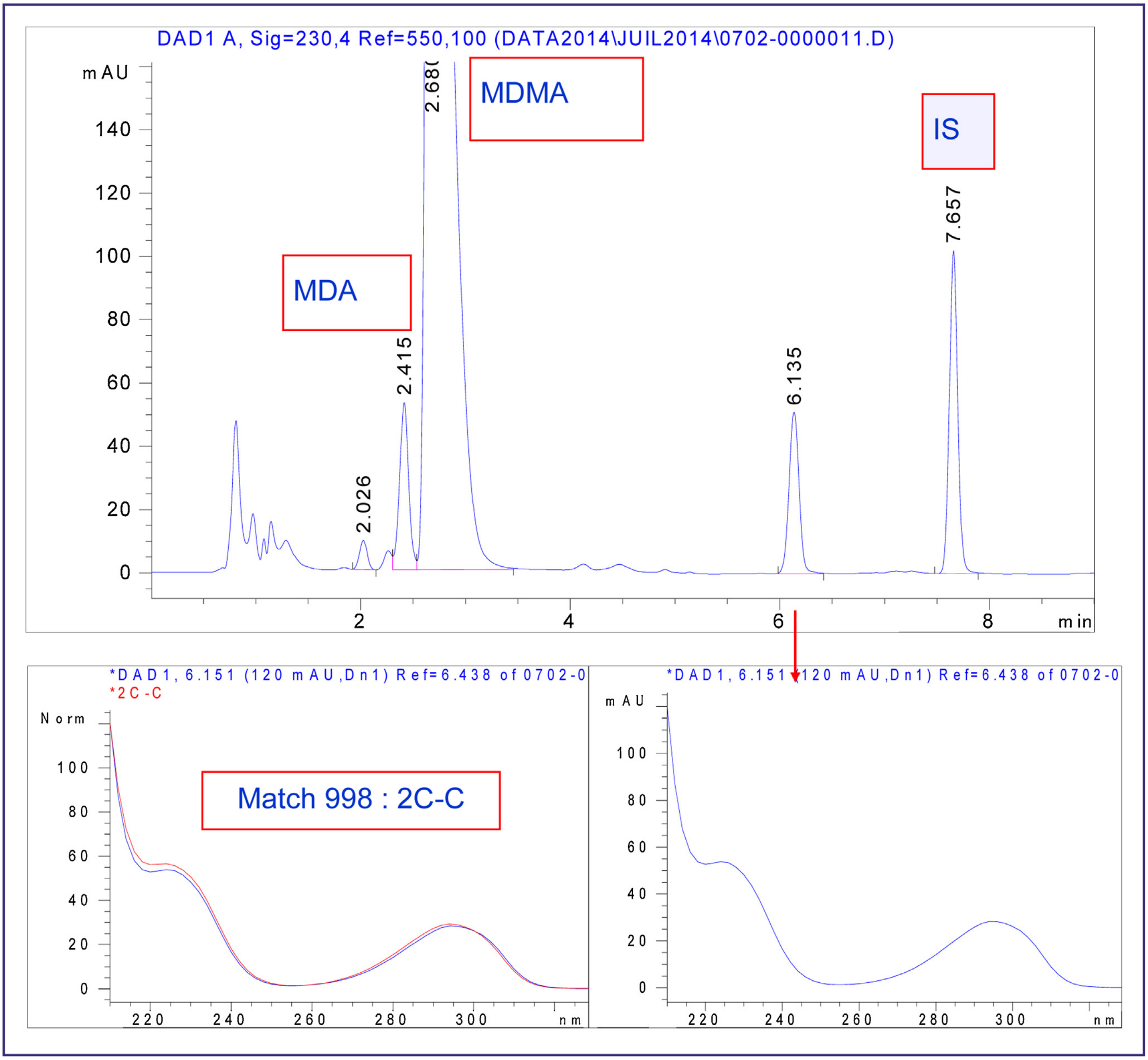

Figure 2. LC/UV-DAD chromatogram of a processed urine sample analysis obtained from patient described in case 1 showing MDMA, MDA, and the unidentified peak (at 6.15 min). Below UV spectrum and the comparison with 2C-C UV spectrum are shown.

Recovery, accuracy and matrix effect were assessed using the procedure of Matuszewski [12]. Five different spiked plasma samples (15, 150 and 750 μg/L) were analysed as described above. Results were compared with those of the same plasma spiked after extraction (matrix effect) and with the same levels of methanolic solutions directly injected onto the LC system on the same day (recovery and overall process efficiency).

Results

Case 1: urinalyses were positive for amphetamines and ecstasy. Blood ethanol was also negative. Urine LSD was negative (routine quantification procedure using LC/MS/MS with LOQ = 0.1 μg/L). The urine screening procedure confirmed the detection of MDMA and its metabolite in addition to an unidentified compound (retention time 6.2 min), with a UV spectrum matching with 2C-C with a retention time shift (5.2 and 6.2 for 2C-C and the unknown compound, respectively) (Fig. 2). The GC/MS analysis following HFBA derivatization allowed MDMA and MDA quantification in urine and plasma (Table 2) and showed a compound with m/z 425, 240, 212, 185 ions. The comparison with 2C-C mass spectrum suggested a structure comparable to this compound with m/z delta of 14 for main ions (Fig. 3).

Case 2: urinalyses were negative for amphetamines and ecstasy. Blood ethanol concentration was 0.13 g/L. Urine LSD was negative. The urine screening procedure showed an unidentified compound (retention time 6.2 min), presenting a UV spectrum matching with 2C-C as it was described in the first patient. Urine analysis using GC/MS method following

Table 2 Summary of main analysis results for urine and plasma samples obtained from the 5 patients.

Patient	Plasma samples		Urine samples		Blood ethanol (g/L)	Other urine analyses
	DOC (μg/L)	Other drugs of abuse (μg/L)	DOC (μg/L)	Other drugs of abuse (mg/L)		
1	10	MDMA = 190, MDA = 14	1300	MDMA = 50, MDA = 2.2	< 0.1	LSD negative
2	13	ND	720	MDMA = 0,05, MDA = 0.02	0.13	LSD negative
3	< 10	Cocaine + BECG = 175, MECG = 14, Ketamine = 100, Norketamine = 250	320	BECG = 16.5, MECG = 4.9, Ketamine = 9, Norketamine = 7	1.28	LSD negative Cannabis positive
4	< 10	ND	300	ND	< 0.1	Cannabis positive
5	18	MPA = 430, Pentedrone = 35	470	MPA = 12.6, Pentedrone = 1.12, AMT = 0.5, Methadone = 0.22	< 0.1	Free morphine = 24 μg/L Codeine = 5 μg/L

MECG: methylecgonine, BECG: benzoylecgonine, MPA: methiopropamine, AMT: alpha methyl tryptamine; ND: no compound detected.

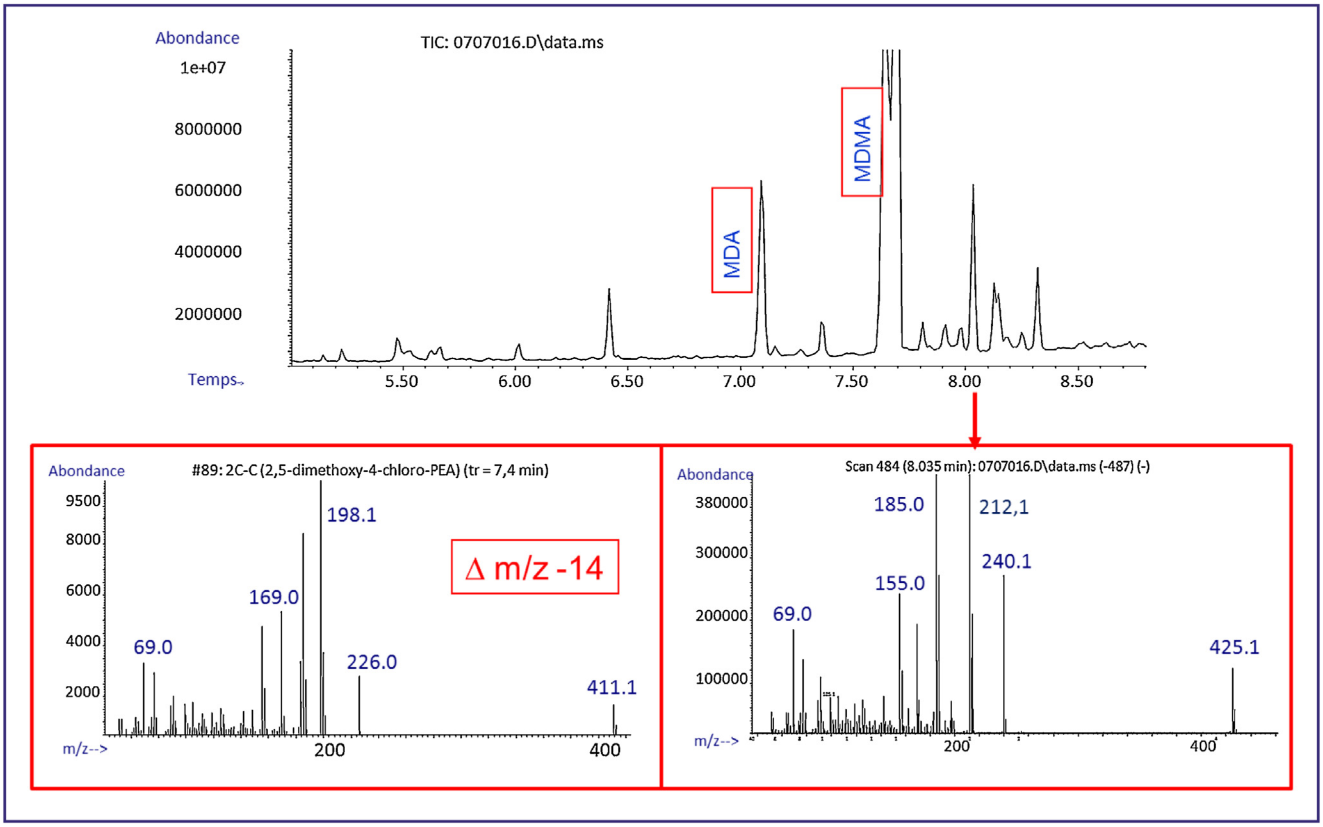

Figure 3. GC/MS Chromatogram of a processed urine sample analysis following derivatization with HFBA obtained from patient described in case 1 showing MDMA, MDA, and the unidentified peak. Below mass spectrum and the comparison with 2C-C mass spectrum are shown.

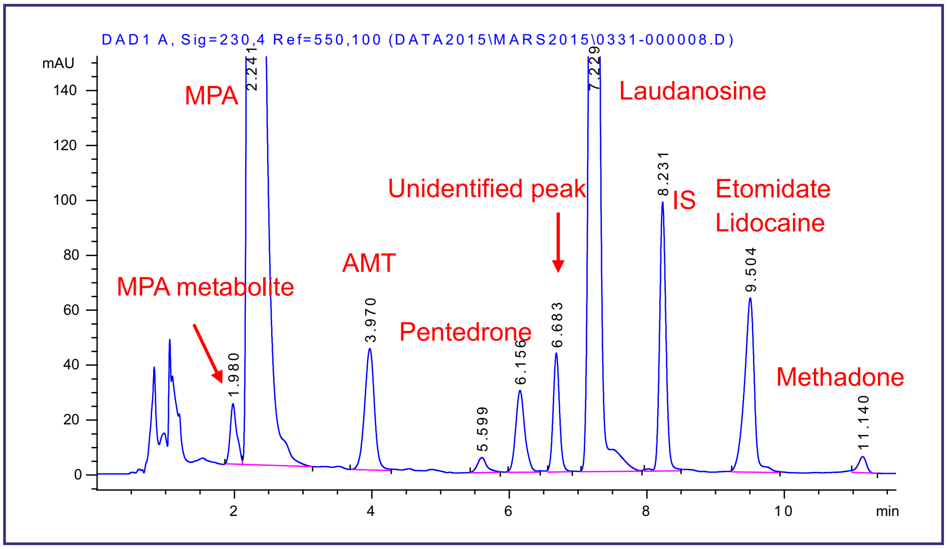

Figure 4. LC/UV-DAD chromatogram obtained after urine sample analysis obtained from patient described in case 5 showing the unidentified peak (at 6.7 min) in addition to other compounds: MPA and its metabolite, AMT, pentedrone, methadone, laudanosine, etomidate, lidocaine.

HFBA derivatization showed the compound with m/z 425, 240, 212, 185 ions.

Case 3: urinalyses were positive for cannabis and cocaine. Blood ethanol concentration was 1.28 g/L. Urine LSD was negative. The urine screening procedure confirmed the detection of ketamine and its metabolites in addition to an unidentified compound (retention time 6.2 min), with a UV spectrum matching with 2C-C identical to that described in previous patients. Routine LC/MS/MS method for cocaine quantification was applied to plasma and urine samples. Cocaine had been detected in plasma in addition to its two metabolites benzoylecgonine and methylecgonine at 175 and 14 µg/L, respectively. Cocaine had been detected in urine in addition to cocaethylene and its two metabolites benzoylecgonine and methylecgonine at 16.5 and 4.90 mg/L, respectively. Cannabinoids quantification had not been carried out. Urine analysis using GC/MS method following HFBA derivatization showed the compound with m/z 425, 240, 212, 185 ions.

Case 4: urinalyses were positive for cannabis and negative for amphetamine. Blood ethanol was negative. Cannabinoids quantification had not been carried out. The urine screening procedure showed also the unidentified compound (retention time 6.2 min) detected in other cases. Urine analysis using GC/MS method following HFBA derivatization showed also a compound with m/z 425, 240, 212, 185 ions.

Case 5: urinalyses were positive for amphetamine (negative for ecstasy), opioids, cannabis and methadone. Blood ethanol was negative. Cannabinoids quantification had not been carried out. The urine screening procedure showed three compounds used during the resuscitation procedure (laudanosine as atracurium metabolite, lidocaine and etomidate), three NPS (methiopropamine, alpha methyl tryptamine, and pentedrone), in addition to methadone (Fig. 4). This procedure showed also the same unidentified compound at retention time of 6.2 min. Urine analysis by GC/MS method following HFBA derivatization showed the compound with m/z 425, 240, 212, 185 ions and confirmed the detection of methiopropamine, alpha methyl tryptamine and pentedrone. Powder sample analysis using the screening procedure showed only one chromatographic peak at retention time of 6.2 min with UV spectrum matching with 2C-C with a retention time shift (5.2 and 6.2 for 2C-C and the unknown compound, respectively). Powder analysis using GC/MS method following HFBA derivatization showed also one peak with characteristic mass spectrum m/z 425, 240, 212, 185 ions. Taken all together, these elements suggested that this unidentified compound is a 2,5-dimethoxy amphetamine and more precisely its chlorinated derivative. The mass spectrum GC/MS analysis following HFBA derivatization can be explained as molecular ion m/z 425 with fragmentation between α and β carbon atoms leading to fragment ion m/z 240 corresponding to the nitrogen moiety and fragment ion m/z 185 for the phenyl part. The ion m/z 212 is the resulted from fragmentation between α carbon and nitrogen (Fig. 5).

To confirm our findings, a LC-MS/MS method for the quantification of 7 DOs was developed and validated (Fig. 6). The best calibration curves fitting was obtained

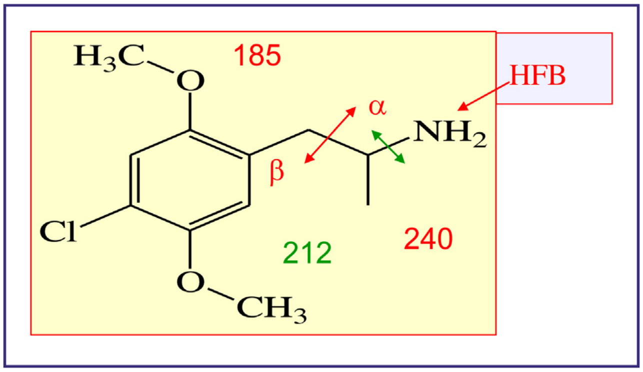

Figure 5. Chemical structure of DOC after derivatization indicating principal ion fragmentation with possible their locations.

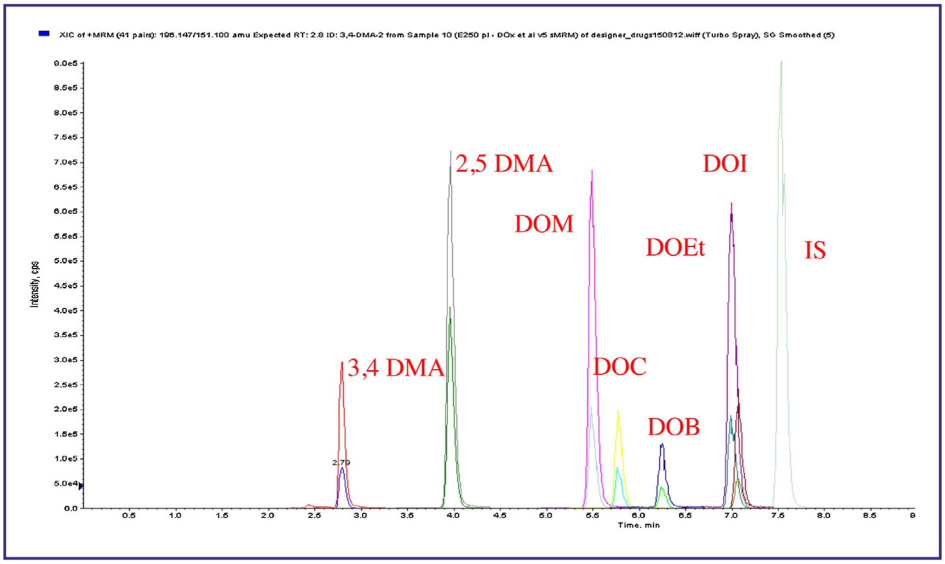

Figure 6. LC-MS/MS chromatogram of plasma sample containing 7 compounds belonging to DO-series (1 mg/L); IS: MethylMilnacipran.

using linear regression for DOC, DOB, DOI and 3,4-DMA ($r^2 > 0.995$) and quadratic equations for DOM, DOEt and 2,5-DMA. The bias was < 15%. Intra-day and inter-day precision (% RSD) were < 6.3 and 6.5%, respectively. Inaccuracy was < 15%. According to the different compounds, the recovery and overall process efficiency were between 85—110% and 77—111% respectively. The matrix effect was between 87—108% and so, there was no ion enhanced or suppression effect. This method was applied to all samples (plasma, urine, and powder) and the analysis confirmed the consumption of DOC by all patients described above and the purity of powder sample obtained in case 5 was found to be > 97%. Quantification results of DOC and other compounds are summarized in Table 2.

Discussion

Five case-reports of consumption of DOC along with a validated quantification method of 7 DOs have been presented. Three reports of confirmed DOC use are available in the medical literature [7—9]. In 2008, Ovaska et al. described the case of a 20-year-old man who consumed a supposed DOI, but paper tabs recovered from other attendees were impregnated with DOC. The patient developed seizures and rhabdomyolysis requiring intensive care unit admission. Toxicological screening confirmed the presence of both DOC and MDMA in plasma and urine without DOC quantification [7]. The 2nd case, described by Burish et al., was a case of a patient who stated that he thought he took LSD and had a urine toxicology negative for amphetamines yet had seizures. The patient's samples screened positive for DOC, which was confirmed by comparison to a reference standard. The serum concentration of DOC in the emergency department was measured to be 3 μg/L approximately 5.5 hours after drug ingestion [8]. The 3rd case, described by Barnett et al., was a fatality due to acute intoxication of a 37-year-old man who was a known methamphetamine abuser. GC-MS screening procedure applied on peripheral blood and urine was positive for DOC only. Quantification analyses showed DOC concertation levels to be 377 μg/L in iliac blood, 3193 μg/L in urine, 3143 ng/g in liver and 683 ng/g in brain [9]. To date, no other clinical case was found in the literature. Meanwhile, Balikova reported two cases of severe intoxication with DOB. Quantification analyses showed DOB concertation levels to be 13 μg/L in the surviving patient, and 19 μg/L in the deceased patient [13]. DOC and its related analogues DOI and DOB are substituted amphetamine derivatives and hence are controlled by the generic Misuse of Drugs Act (1971) in the UK. DOC and DOI are controlled as Schedule I substances in Denmark and DOC is listed in Analge I in Germany. However, in all other European countries, including France, these compounds are not covered under current drug legislation; hence possession and supply remain legal. It is to be noticed that in 3 cases documented in this paper, LSD-like blotting papers were mentioned either by patients or by attendees. In other two cases, LSD or LSD derivative were believed to be ingested by patients. Burish et al. also reported that the patient had stated that he thought he took LSD [8]. This may be due to an emerging use of DOC as 'legal' substitute to LSD and lead us to suggest that this compound, in addition to DOI and DOB, should be controlled and scheduled in all European countries.

For the toxicological laboratories concerned by both clinical and forensic analyses of NPS, the challenge is often to obtain the pure reference standards but also to differentiate the structurally related compounds. Nevertheless, when toxicological screening is undertaken with appropriate expertise and pure reference standards are available, novel drugs may be detected as it has been proven in the case described by Ovaska, in which the patient believed that he has ingested DOI, but toxicological screening confirmed that he had, in fact, ingested DOC. As the matter of fact, we share the point of view supported by other published reports which have suggested that systematic toxicology screening in centres treating large number of patients with recreational drug toxicity may help to determine evolving epidemiological trends in recreational drug availability, use and toxicity [7,14,15].

Disclosure of interest

The authors declare that they have no competing interest.

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CASE REPORT

The development of new psychoactive substances in France

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Received 2 December 2015; received in revised form 5 May 2016; accepted 9 May 2016
Available online 9 July 2016

KEYWORDS
Designer drugs/analysis;
Street drugs/analysis;
Gas chromatography—mass spectrometry/methods;
France

Summary If our European neighbours have been involved in the fight against new psychoactive substances (NPS) for several years, the seizures from our police forces have been rare. Only the laboratories of French customs analysed large amounts of NPS. Nevertheless, in the last three years, an increased number of French toxicologists have reported detection of NPS use. On the ground, because of the posed problems, police officers attempt to make it part of their jurisdiction and seize the substances. Thus, from our laboratories, the seizures could bring much information about the NPS phenomenon. From seizures submitted to our laboratory, all were analysed according to standard analytical strategy defined in our quality plan. Between 2013 and 2014, 62 seizures of NPS were analysed in our laboratory: 4 seizures in 2013 and 58 seizures in 2014, showing a sudden increase. Synthetic cannabinoids were the main substances identified (15 different compounds detected), five different phenylethylamines, four different cathinones, two tryptamines, two new benzodiazepines, methoxetamine, methiopropamine, mCPP and dimethylaminoethanol were also found. If the proportion of NPS seizures remains low in comparison with the more ''classical'' drugs of abuse in our laboratory, the dramatic increase in requests from investigators expresses the expansion of NPS throughout France in all likelihood.

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http://dx.doi.org/10.1016/j.toxac.2016.05.053
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Introduction

Detection of new psychoactive substances (NPS) in seizures remains uncommon even for French forensic laboratories that annually analyse thousands of forensic drug exhibits. Only the French customs have reported identification of these items on a more regular basis. [1,2]. Since 2000, Système d'identification national des toxiques et substances: National System of Identification of Poisons and Substances (SINTES/NSIPS) from the Observatoire français des drogues et toxicomanies/French Monitoring Centre for Drugs and Drug Addiction (OFDT/FMCDDA) has reported the arrival of new identified substances in France [3,4]. However, the full extent of the NPS problem remains unknown and continues to be difficult to assess. In this context, the emergence of synthetic cannabinoids (SC) in Mayotte [5] surprised us at the beginning of 2014 but made us aware of the existence of a NPS market in France.

For the forensic science laboratories of the French police forces (French police and French gendarmerie), all the materials analysed consisted of sealed exhibits. These seizures were either directly obtained by the investigators after arrest of a potential trafficker or user, or found beside an intoxicated person or, in some cases, submitted by the customs officers as part of legal proceedings. These submissions do not fully represent the reality of the French NPS market but the findings from the analyses have aided in obtaining a prosecution.

Alongside colleagues from the French police, the Forensic Toxicology Unit (FTU) of the Forensic Science Laboratory of the French Gendarmerie (Institut de recherche criminelle de la gendarmerie nationale) plays its part in the analyses of suspected narcotic seizures and consequently in the detection of suspected NPS seizures. Because the FTU's influence is more national than regional as we receive requests from all over France, establishing the number of NPS cases submitted to our laboratory and the discussion on their possible or observable trends seemed an interesting factor warranting investigation. These findings will complete the previously reported observations in Mayotte [5].

Equipment and methods

Materials

The 63 seizures consisted of powders of different colours (see Fig. 1), tablets, and finely chopped material.

Equipment and reagents

An Ionscan 400B (Smiths Detection, Vitry-sur-Seine, France) and an Agilent 5975 GC/MSD System (Agilent Technologies, Les Ulis, France) equipped with a ZEBRON ZB-5MSi 30 m × 0.25 mm × 0.25 μm purchased from Phenomenex (Le Pecq, France) were employed in this work.

Methanol and acetonitrile of analytical grade were purchased from Carlo Erba (Val de Reuil, France), Sil-Prep™ Alltech™ Silylation Reagents from Chromoptic (Courtaboeuf, France).

Methods

The seizures were analysed, following our standard analytical strategy and the test methods employed were compliant within the scope of our accreditation (N° 1-1916 rev.4). Powders were dissolved in methanol and in acetonitrile at 1 g/L; plant materials were incubated at 1 g/L in methanol. Supernatants were split for multiple analyses and an aliquot was dried and derivatised by Sil-Prep™.

The methanolic supernatants were analysed by the ion mobility spectrometer (IMS). For this screening, 5 μL of the methanolic solutions were transferred to a swab, dried and analysed with IMS by following the manufacturer's instructions. Because of the nature of the seizures, and consequently the lack of identification in this first step using IMS, the second step consisted of a general unknown screen by GC—MS.

For this screening, the methanolic and acetonitrile supernatants as well as the derivatised solutions were used. Only 1 μL of each of the solutions was injected and analysed using the instrument parameters previously reported. The observed mass spectra were compared to the spectral libraries from the drug working group of ENFSI (ENFSI-DWG) and the scientific working group for the analysis of seized drugs (SWGDRUG [6]). Methods have been previously reported [5].

Results

Between 2013 and 2014, 62 seizures of NPS were analysed in our laboratory: 4 seizures in 2013 and 58 seizures in 2014, showing a dramatic increase in the last year. Synthetic cannabinoids (SC) were the main substances identified (15 different compounds detected (see Table 1); 3 seizures in 2013 and 21 in 2014, SC are mainly alone, sometimes associated with other SC and once with ethylphenidate [IUPAC name: 2-phenyl-2-piperidineacetic acid, ethyl ester]), we also identified five different phenylethylamines (ethylphenidate, MDAI [6,7-dihydro-5H-indeno[5,6-d]-1,3-dioxol-6-amine], 3,4-dichloromethylphenidate [(2R)-2-(3,4-dichlorophenyl)-2-piperidineacetic acid, methyl-ester], camfetamine [N-methyl-3-phenylbicyclo[2.2.1]heptan-2-amine], fencamfamine [N-ethyl-3-phenyl-norbornan-2-amine]; 15 seizures in 2014, mainly alone, sometimes associated with other phenylethylamines and once with 5F-AKB-4), four cathinones (MDPV [1-(1,3-benzodioxol-5-yl)-2-(1-pyrrolidinyl)-1-pentanone], methylone [1-(1,3-benzodioxol-5-yl)-2-(methylamino)-1-propanone], ethcathinone [2-(ethylamino)-1-phenyl-1-propanone], 4-methylethcathinone [2-(ethylamino)-1-(4-methylphenyl)-1-propanone]; 1 seizure in 2013 and 6 in 2014, mainly alone, although once identified as a mixture and once with methoxetamine [2-(ethylamino)-2-(3-methoxyphenyl)-cyclohexanone]), two tryptamines (5-MeO-DALT [5-methoxy-N,N-di-2-propen-1-yl-1H-indole-3-ethanamine] and αMT [2-methyl-1H-indole-3-ethanamine]; 2 seizures in 2014), two benzodiazepines (etizolam [4-(2-chlorophenyl)-2-ethyl-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo[4,3-a][1,4]diazepine] and diclazepam [7-Chloro-5-(2-chlorophenyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one];

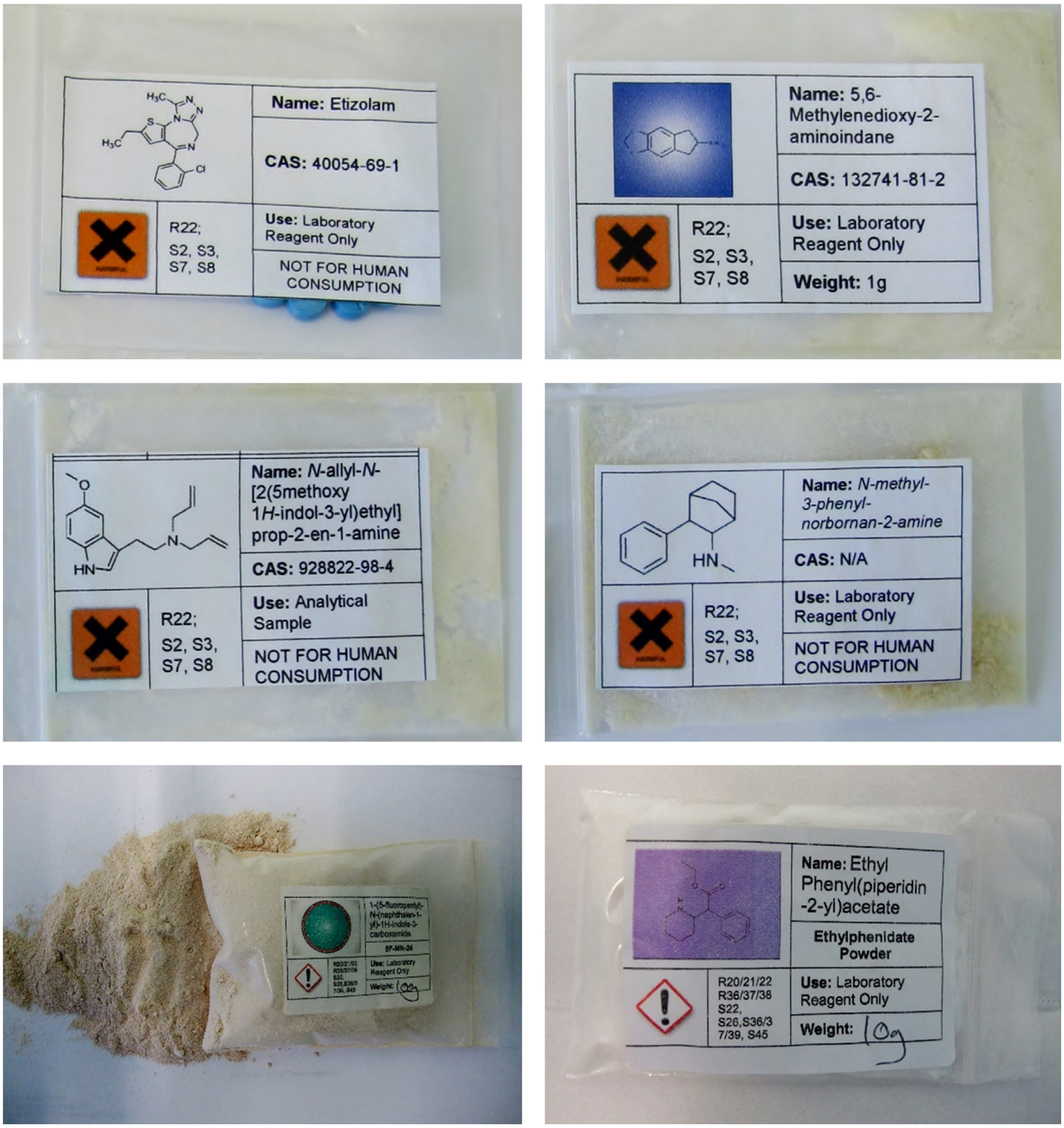
Name: Etizolam
CAS: 40054-69-1
R22;
S2, S3,
S7, S8
Use: Laboratory Reagent Only
NOT FOR HUMAN CONSUMPTION
Name: 5,6-Methylenedioxy-2-aminoindane
CAS: 132741-81-2
R22;
S2, S3,
S7, S8
Use: Laboratory Reagent Only
Weight: 1g
Name: N-allyl-N-[2(5methoxy 1H-indol-3-yl)ethyl] prop-2-en-1-amine
CAS: 928822-98-4
R22;
S2, S3,
S7, S8
Use: Analytical Sample
NOT FOR HUMAN CONSUMPTION
Name: N-methyl-3-phenyl-norbornan-2-amine
CAS: N/A
R22;
S2, S3,
S7, S8
Use: Laboratory Reagent Only
NOT FOR HUMAN CONSUMPTION
1-(5-fluoropentyl)-N-(naphthalen-1-yl)-1H-indole-3-carboxamide
5F-MN-24
Use: Laboratory Reagent Only
Weight: 10g
Name: Ethyl Phenyl(piperidin -2-yl)acetate
Ethylphenidate Powder
R20/21/22
R36/37/38
S22,
S26,S36/3
7/39, S45
Use: Laboratory Reagent Only
Weight: 10g

Figure 1. Examples of non-packaged bags of NPS seized in 2014, explicitly annotated with formula and chemical name.

6 seizures in 2014), methiopropamine [N,2-dimethyl-2-thiopheneethanamine] (6 seizures in 2014), mCPP [1-(3-chlorophenyl)piperazine] (once in 2014), methoxetamine (once in 2014) and dimethylaminoethanol [2-(dimethylamino) ethanol] (twice in 2014) (Table 2). All of these seizures originated from different areas of France, both mainland and overseas.

Discussion

Our results suggest an increase in NPS seizures in France. This observation is corroborated by the apparent increase of reported NPS cases in STUPS©: between 2013 and 2014, the percentage of NPS rose from 1.7% to 2.9% amid the seizures analysed by the forensic institutes of the French police forces. At the same time, the number of articles from French authors with ''designer drugs'' (official MeSH term) as keywords or term also increased. (Fig. 2). However, when comparing to other European countries, the traffic of NPS seemed to be different, in fact it was found to be lower. In France, SC were found to be less diversified than identified across Europe. In 2013, SC represented 46% of the total NPS seizures in Europe [7] but only 6% in France (STUPS©). In fact, the two main NPS families seized and analysed by the French police forces in 2013 and 2014 were the cathinones and the phenylethylamines (STUPS©). In Mayotte however, where there were many SC seizures, was an exception to this [5].

It should be noted that the French NPS market is very different from some other European countries, for example, the United Kingdom or Poland [4]. For the Polish Government, the fight against the use of NPS has become an important challenge: with around 1400 NPS shops on their

Table 1 Synthetic cannabinoids detected in the seizures.

SC identified	SC identified
UR144 [(1-pentyl-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)-methanone]	5F-AKB-48 [1-(5-fluoropentyl)-N-(tricyclo[3.3.1.$1^{3,7}$]dec-1-yl)-1H-indazole-3-carboxamide]
AB FUBINACA [N-[(1S)-1-(Aminocarbonyl)-2-methylpropyl]-1-[(4-fluorophenyl)methyl]-1H-indazole-3-carboxamide]	5F-MN-24 [1-(5-fluoropentyl)-N-(naphthalen-1-yl)-1H-indole-3-carboxamide]
FUB-PB-22 [1-[(4-fluorophenyl)methyl]-1H-indole-3-carboxylic acid, 8-quinolinyl ester]	AM2201 [1-[(5-fluoropentyl)-1H-indol-3-yl]-naphthalenyl-methanone]
THJ2201 [[1-(5-Fluoropentyl)-1H-indazol-3-yl]-1-naphthalenyl-methanone]	5F-PB-22 [1-(5-fluoropentyl)-1H-indole-3-carboxylic acid, 8-quinolinyl ester]
BB22 [1-(cyclohexylmethyl)-1H-indole-3-carboxylic acid, 8-quinolinyl ester]	JWH022 [1-naphthalenyl[1-(4-penten-1-yl)-1H-indol-3-yl]-methanone]
THJ018 [1-naphthalenyl(1-pentyl-1H-indazol-3-yl)-methanone]	AM1220 [[1-[(1-methyl-2-piperidinyl)methyl]-1H-indol-3-yl]-1-naphthalenyl-methanone]
JWH 018 adamantyl carboxamide [1-pentyl-N-tricyclo[3.3.1.1 3,7]dec-1-yl-1H-indole-3-carboxamide]	MAM-2201 [[1-(5-fluoropentyl)-1H-indol-3-yl](4-methyl-1-naphthalenyl)-methanone]
STS135 [1-(5-fluoropentyl)-N-tricyclo[3.3.1.$1^{3,7}$]dec-1-yl-1H-indole-3-carboxamide]	

territory until 2010, Polish people are very exposed. Annually, NPS have caused more than 1000 cases of medical intervention and at least 3 deaths since 2013 in Poland [8,9]. In the same period, the UK identified only 250 headshops [10] but at least 19% of the NPS online shops reported by the EMCDDA are British, when there were only 3% of sites originated in Poland [11]. Finally, the NPS-related deaths were more abundant in the UK with up to 52 cases in 2012 [12]. In comparison, in France, until 2014 no public headshops had been observed and the hospitalisations and the deaths after NPS overdose had appeared rare or had not been reported. From these differences, it may be deduced that France had been untouched by NPS.

In 2014, the NPS products showed great variation in their contents. As an example, tablets of dimethylaminoethanol (DMAE) or Deanol (INN) have been identified amongst the seized NPS. In France, this substance has been sold in mixtures as an antasthenic (ACTI 5 or Débrumyl) for a while. When looking for a reason to their presence amidst the seizures, one finds that recreational-drug users have tried the DMAE, on its own or as a mixture since the 2000s [13]. There appear to be several different reasons for this finding: improvement of memory, learning or activity; induction of lucid/vivid dreams [14,15]; as a source of acetylcholine when a racetam is used (to enhance racetam effects or to prevent choline depletion) or anti-ageing [16]. As with other nootropics, DMAE (which remains unregistered) completes and complicates the long list of NPS compounds identified in seizures.

It appears, anecdotally, that dealers and users have developed a sense of impunity, as they are convinced of the lack of power of arrest by police officers. This is thought

Table 2 Summary of results.

Family	Number of seizures	When associated together	When associated with another family
Benzodiazepine	6	—	—
Cathinone	7	1	1 (methoxetamine)
Phenylethylamine	16	2	1 (synthetic cannabinoid)
Synthetic cannabinoid	24	5	1 (phenylethylamine)
Tryptamine	2	—	—
Others	9	—	1
Methiopropamine	6	—	—
DMAE	2	—	—
Methoxetamine	1	—	1 (cathinone)
Piperazine	1	—	—

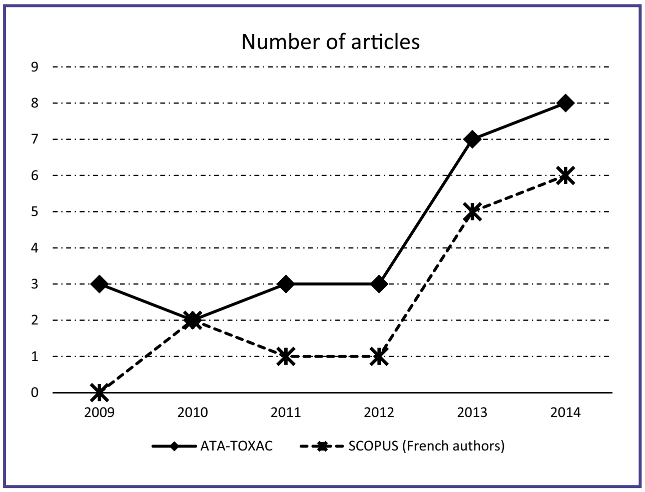

Figure 2. Evolution of the number of articles from French authors in ATA and TOXAC (solid line) or on SCOPUS (dotted line) between 2009 and 2014.

to be the case because some of the substances were not yet classified as narcotics and/or were undetectable with screening tests available at the time. NPS are still readily available on the Internet, and in France, they seem to be sold in a relatively pure form, in simple packaging, explicitly annotated with formula and chemical name (Fig. 1). However, the apparent legality and elaborate packaging may have confused the police officers who may previously have only seized the most ''home-made'' looking packages. Training of all the officers involved in the punitive system is required for better identification at source but the very nature of the NPS products complicates the problem.

The broad range of products complicates the possible response by the authorities. The police officers have to be educated with respect to the NPS problem (appearance and the forms in which they may present). On the ground, the police officers need screening tests and dogs able to detect all the chemical families of NPS, as well as the more classical street drugs. Within the laboratories, forensic toxicologists are also required to have fully developed, wide-screening methods. If we do not have these basic requirements met, the fight otherwise, will be vain and unproductive.

In order to prosecute for narcotic trafficking or use, it is necessary that the NPS must be controlled. The speed of their arrival on the market nevertheless makes the substance-by-substance registration more difficult and inefficient. In this regard, the legal system is not as reactive as the dealers. To circumvent this problem, several governments have decided to control the substances by having generic laws which cover many of the major classes of NPS compounds currently identified in seizures. The best French examples of these generic laws are the registration of the cathinones and of some SC: a chemical basic structure and its numerous possible substituents are currently controlled. This approach generalises the registration and tries to prevent the appearance of new NPS however, on the ground and in the laboratories, from a practical point, all of the teams involved are not currently ready and able to detect unknown substances that are already registered.

Conclusion

Although the trafficking of NPS in France is much less of a problem in comparison to the classical narcotics, an observed increase has been seen since 2013, as the number of seizures submitted to the laboratory can attest to. However, the French NPS market is still different to that identified in other European countries, as SC were not the commonly seized substances (except in the case of Mayotte). The presence of DMAE amongst the seizures confirms that this market is well diversified what complicates the legal solutions.

In 2015, the French government registered several new substances, amongst them was ethylphenidate and 7 families of SC. The full consequences of this classification have yet to be seen, but it is important to note that the classified SC families do not contain all of the currently observed circulating substances.

Finally, the best defence against NPS trafficking appears to be this generic law, even if it leaves the toxicologists dealing with unknown narcotics. At this point in time, it is difficult to say that toxicologists are ready to detect the influx of the wide range of NPS products. However, we must arm ourselves as best we can with the tools and information we have available to us. In any case, the French synthetic substance market might have changed in 2015: amphetamine, methamphetamine and ecstasies appear to be re-emerging.

Disclosure of interest

The authors declare that they have no competing interest.

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Toxicologie Analytique & Clinique (2017) **29**, 96—100

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CASE REPORT

''NRG-3'': What's in this?

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Received 28 October 2016; received in revised form 14 December 2016; accepted 15 December 2016
Available online 16 January 2017

KEYWORDS
Cathinones;
MDPV;
Harm reduction;
Toxicity

Summary The supply of recreational drugs has changed in the early 2000s and users increasingly buy cathinones over the Internet. There is a potential for significant toxicity associated with their use. Cathinone named ''NRG-3'' seems to be the most popular in Parisian CEIP data: the adverse events related to use of NRG-3 from 2011 to 2015 are reported. We aim at determining the chemical composition of NRG-3 available over the Internet and whether products differ depending on retailers. Three powders labelled ''NRG-3'' were purchased on July 2012 from three different Internet sites, moreover one capsule and one powder labelled ''NRG-3'' was given by drug users. These were analysed by LC—MSMS to determine active ingredients. The cathinones class was detected, without active adulterant. A great inconsistency in the qualitative and quantitative composition of products bearing identical labelling ''NRG-3'' was shown. We found two different single components: methylene dioxypyrovalerone (MDPV) and α-pyrrolidinopentiophenone (α-PVP) with mixture preparations in (3/5) samples. α-PVP varied from 4 to 80% and MDPV varied from 0 to 70%. There was significant variation of the products contained in NRG-3. This variation could be of clinical significance as the NRG-3 can be associated with worrisome toxicity. Marked agitation with violent and unpredictable acts is reported with MDPV: this molecule has a higher ability to cross the blood brain barrier because the pyrrolidine ring confers a low polarity. Given the plethora of NPS, proper drug identification is core essential for harm reduction associated with empowered users through education.

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http://dx.doi.org/10.1016/j.toxac.2016.12.005
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Introduction

The supply of recreational drugs has changed in the early 2000s [1]. Users increasingly buy ''new psychoactive substances'' (NPS) sold variously as 'legal highs', 'bath salts' or 'research chemicals', under ambiguous names such as 'K2', 'Spice' or 'NRG-1'. Most of the time, they are pharmacological analogues of some prohibited compounds, such as amphetamines or cannabis and are available from online vendors or in so-called 'head shops'. For 4 consecutive years (2011—2014), the European early warning system has detected a high record of NPS and a growing number of online vendors [2,3]. In 2014, 480 substances including stimulants, synthetic cannabinoids, hallucinogens, dissociatives and sedatives were being monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [3,4]. The United Nations Office on Drugs and Crime (UNODC) defines ''new psychoactive substances'' as ''substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat''. The term ''new'' does not necessarily refer to new inventions — several NPS were synthesized 40 years ago — but recently emerged on the market and are not scheduled by Conventions. The term NPS also applies to plants, which have been recently diverted from their legitimate ritual or traditional use (e.g. kratom) [5]. By December 2015, over 644 NPS had been reported by 102 countries to UNODC, showing a stark and unprecedented increase in NPS emergence since 2008 [6]. The largest groups of NPS are synthetic cannabinoids and synthetic cathinones.

Cathinone is an alkaloid found in the khat plant (*Catha edulis*). Chewing the khat leaves is popular in Somalia and Yemen (east Africa and Arabian Peninsula, respectively) due to its stimulant properties. Synthetic Cathinones are β-ketoamphetamine derivatives, which possess a ketone group at the β carbon position of the amphetamine backbone [7]. There is a potential significant toxicity associated with their use. Users openly speak of Synthetic Cathinones as ''legal high''. Since July 2012 [8], in France, all synthetic drugs from the cathinones family have been banned and listed as narcotics. Indeed, despite the 2012 law, a legislative directive introduced the generic approach to the regulation of NPS (Ministry of Social Affairs and Health, 2012); that is, criminalisation refers to a group or family of products rather than a specific substance [9]. Among drug users, it could be commonly observed a misconception that these compounds are ''safe'', with a great purity [10—12]. The packaging of these does not specify the composition of the active constituents. Cathinones named ''NRG-3'' seem to be very popular among users in Parisian population [13], especially in MSM (men who have sex with men). The Parisian centre CEIP-A has collected notifications of adverse events with NRG-3 from 2012 to 2015. NRG-3 is known to be a mix of different synthetic cathinones. NRG-3 users report us some variation of effects according to purchases or batches. In the present study, we aim at determining the chemical composition of NRG-3 available over the Internet, in order to develop specific interventions in the area of harm reduction of cathinones use.

Methods

Samples

Three powders labelled ''NRG-3'' were purchased in July 2012 from three different Internet websites. One capsule and one powder labelled ''NRG-3'' were given by drug users to their practitioner in November 2012 but these samples did not have their original package.

Chemicals and reagents

Formic acid and methanol were all high-performance liquid chromatography (HPLC) grade and were supplied by Sigma—Aldrich (Paris, France). 3,4-methylenedioxypyrovalerone (MDPV) and its deuterated analog, MDPV-d8, were purchased from Toronto Chemicals Inc (Toronto, Ontario, Canada). α-pyrrolidinopentiophenone (α-PVP) was purchased from Cayman Chemical Company (Ann arbor, Michigan, USA). LSD-d3 was from LGC standards (Molsheim, France). Ultra-pure water (18 MΩ) was obtained using an Elix apparatus (Millipore Corp., Molsheim, France).

Analytical methods

After macroscopic description, one 10 mg aliquot of each sample was solubilized in 1 mL of methanol, sonicated for 5 minutes, and subsequently 1:10,000 diluted in methanol. Qualitative analyses were performed by Gas chromatography and Mass spectroscopy (GC-MS) using a single quadrupole DSQ 2 mass spectrometer operated in full scan (m/z: 50→450) (ThermoFisher Scientific, San jose, Ca, USA). The chromatographic separation was performed using a CP-Sil 8CB-MS column (0.25 μm, 25 m × 0.25 mm) (Agilent technologies). The total analysis run time was 25 min.

For quantification, an online TurboFlow-LC—MS/MS system Aria TLX-1 LC system coupled to TSQ Quantum Ultra triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used. An Aria Transcend TLX-I system (ThermoFisher Scientific, San Jose, USA) consisting of two Accela 600 high-pressure quaternary pumps (one loading pump for the TurboFlow system and one eluting pump for the analytical column system), valve interface module, and CTC PAL autosampler was used. Chromatographic separation was achieved on a Supelco HSF5 analytical column (100 × 4 mm, particle size: 3.5 μm). The total analysis time was 9.5 min.

A Quantum Ultra triple-quadrupole mass spectrometer with an electrospray ion source (ThermoFisher Scientific, San Jose, USA) was used for detection. Analyses were carried out in the positive ion mode using multiple reaction monitoring (MRM) specific for MDPV, a-PVP and its deuterated analog.

As mentioned, all samples were first qualitatively analyzed by gas chromatography/mass spectrometry (GC-MS) combination. In a second step all compounds (active ingredients and potential active adulterants) detected by GC-MS were quantified by using a validated liquid chromatography-tandem mass spectrometry (LC—MS/MS) assay.

In another part, we study in the Parisian addictovigilance database the adverse events related to use of NRG-3 from 2011 to 2015 (a five-year period).

Table 1 Packaging information.

	Sample 1 — Supplier 1	Sample 2 — Supplier 2	Sample 3 — Supplier 3
Trade name	NRG-3	Research Chemical (NRG-3)	Energy-3 (NRG-3)
Chemical classification	(2α, 3α) epithio-17α-methyl 17β-ol-N-benzyloxycarbonyl-d-proline	3 (E-3, etoxyphenyl)-2-(ethylamino) cyclohexanone	
Sample weight	001 g	1 g	1 g
Skeletal formula	Yes (PVP)	No	No
Hazard pictogram	Yes	No	No
CAS number	136741-83-2 (false)	No	No
Molecular mass	177,1998 g/mol (false)	No	No
Warning	Only permitted for use a laboratory reagent. Not for any in vivo testing	Commercial sample for research purposes only	Strictly not for human consumption

Results

Information available on packaging is shown in Table 1. For example, (2α, 3α) epithio-17α-methyl 17β-ol-N-benzyloxycarbonyl-d-proline appears to be a counterfeit name to evade the laws. Moreover, the notion ''Warnings'' on the packaging is thought to allow circumventing the law and permits the product to be conveniently delivered by post. For the two given samples, they were collected without original packaging (no information).

Some qualitative and quantitative variations were found in these products while all were named NRG-3. The powder found to contain: two α-pyrrolidinopentiophenone (α-PVP) samples and three mix of α-PVP with methylenedioxypyrovalerone (MDPV), which are both synthetic stimulant drugs of the banned cathinones class. The purity of α-PVP varied from 4 to 80% and MDPV ranged from 0 to 70% (see below Figs. 1 and 2). These two drugs of cathinones class were detected without any active adulterant.

In Parisian Addictovigilance database, twenty-nine cases of NRG-3 abuse or dependence were notified. NRG-3 represented 23% of all cathinones notifications (mephedrone 24%, 4-MEC 23% and MDPV 15%). NRG-3 users were men (100%), with mean age of 38 years (21 to 58 years), showing having cathinones abuse in sexual context (22/29). The NRG-3 was most commonly administered by intravenous injection (70%): an increasingly popular practice, called ''slam'', among Parisian men who have sex with men (MSM). The SLAM comprises three characteristics: injection, sex party and psychostimulant drug. This is an especially worrisome trend since the prevalence of HIV infection in our cases were 62% (18/29) with a high level of co-infection HIV/Hepatitis C (66% of VIH). In 34% (10/29), they snorted NRG-3 powder. In five cases, route of use was oral and in two cases, it was inhaled. NRG-3 users tended to be experienced by polydrug users (62%, 18/29).

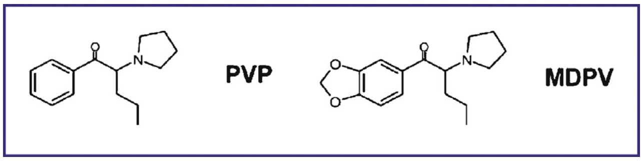

Figure 1. Pyrrolidine derivatives group.

Thirteen cases presented psychiatric disorders: psychotic symptoms (9/29), depression/anxiety (6/29), suicidal ideas or attempt (3/29) and mania (2/29). Two cases presented a sexual behaviour disorder with hypersexuality. A significant minority of patients have presented hallucinations (3/29). Moreover, we report two deaths due to violent suicide (firearm).

Somatic complications were described in 5 cases with various patterns of symptoms caused by direct (skin rash, temporo-spatial disorientation) or indirect (peritonitis, abscess, seroconversion complications [14]) toxicity of NRG-3.

Nineteen patients (19/29) presented a cathinones use disorder with craving, and withdrawal syndrome.

Discussion

EMCDDA reports the increasing concern about the emergence of a new trend among some sub-groups of gay men involving the injection of illicit drugs, including cathinones and methamphetamine, in a practice known as 'slamming' in 2015 [15]. Likewise, injecting cathinones use is reported in France, in a recent study that has developed a scientific approach to obtain information through the chemical analysis of residues in syringes [16]: the analysis results showed that 23% of the syringes contained 4-methylethylcathinone ($n = 3489$ syringes).

Seroconversion complications with cathinones are discussed: Although the high incidence of HIV infection in illicit drug abusers is primarily due to high-risk activities such as needle sharing and unprotected sex, several studies have demonstrated that cocaine enhances the rate of HIV gene expression and replication by activating various signal transduction pathways and downstream transcription

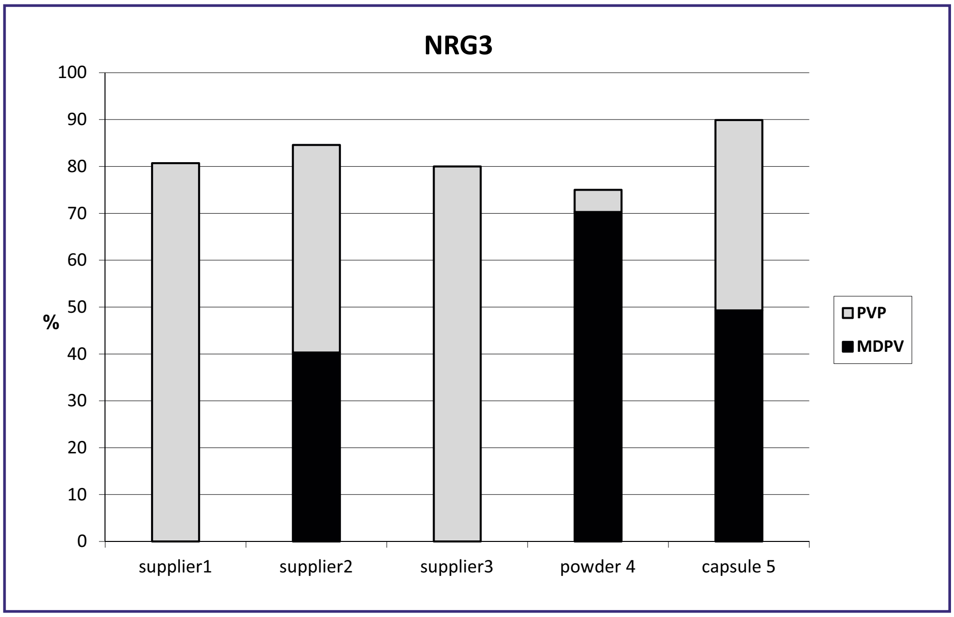

Figure 2. Composition of samples.

factors [17]. Moreover, studies report that drugs of abuse, such as cocaine, amphetamine, methamphetamine, heroin and morphine, modulate those mechanisms to upregulate HIV transcription and eventually HIV replication [18].

The synthetic cathinones are able to inhibit monoamine uptake transporters producing a decreased clearance of the neurotransmitters from the synapse. Furthermore, they may release biogenic amines from intracellular stores (inhibition of dopamine and norepinephrine reuptake transporters and, to lesser extent, serotonin transporters). The perceived effects of cathinone stimulants are dose-related and include euphoria, increased energy, and increased libido [19].

All synthetic cathinones are generally less able to cross the blood brain barrier than amphetamines because the beta-keto group causes an increase in polarity [20]. In contrast, pyrrolidine derivatives group including MDPV and α-PVP displays a higher ability to cross the blood brain barrier because of the pyrrolidine ring, which confers a low polarity [21]. A high agitation is reported with bath salts containing MDPV [22]. Police reports indicate frequent violent and unpredictable acts from people under the influence of MDPV. Several deaths were related to MDPV [23]. These results suggest that MDPV could have a strong potential for compulsive use and addiction in humans [20].

The variation of composition represents an interesting approach of harm reduction related to the use of NPS: specific attention should be devoted to the drug analysis for NPS users. The true content of the product can be very different from the one indicated on packaging [24]. A study reports that the cathinones sold on websites contain benign active ingredients (such as phosphates, magnesium salts) but also caffeine or local anaesthetics [25]. Another study underlines that 25% of a legal high brand name product has changed over a 6-month period [26]. Brandt et al. [27] report an analysis of thirteen NRG-1 samples (claming to contain naphyrone): only one shows analytical results consistent with naphyrone. Otherwise, Alotaibi et al. [28] showed there are risks from the pharmaceutically active cutting agents like benzocaine.

Providing information about product testing is a tool of harm reduction for drug users. Faced with widespread health problem caused by NPS, analytical techniques such as liquid chromatography — mass spectrometry (LC—MS) or gas chromatography — mass spectrometry (GC-MS) should be used more often for specific detection and quantitation in order to develop knowledge of NPS toxicity and pharmacology.

The attraction for the synthetic cathinones is due, in a large part, to the decreased availability and purity of the more typical drugs of abuse. Indeed in 2005, a worldwide shortage of MDMA, the original chemical found in pills sold as ''ecstasy'', led to a decline of the ''ecstasy'' market. Nevertheless, the continuing demand for ''ecstasy'' led to the use of alternative chemicals to MDMA [6]. Moreover, cathinones are relatively inexpensive (20 € per 1 g) and are easily available on Internet. In France today, the drug analysis device as harm reduction is not very developed.

Conclusion

There is a significant variation of the products contained in NRG-3. This variation could be of clinical significance since the NRG-3 can be associated with worrisome toxicity. Marked agitations with violent and unpredictable acts are reported with MDPV [8]. The name is insufficient information for buyers and consumers to be confident as for their exact content. The variability of active ingredients concentration and the difficulty of predicting the actual pharmacological and toxicological effects related to the simultaneous consumption of different psychoactive ingredients contained in single product are worrying elements. The variation could increase the risk of unwanted effects or lead to acute toxicity and even to death. We point out, first, the lack of publications on variability in composition of NPS, and secondly, researches on

NPS used as cutting agent for traditional drugs. The onward evolution of NPS requires an effective monitoring of purity and the drug checking procedures must be carried out with an official approval. Given the plethora of NPS, proper drug identification is core essential for harm reduction associated with empowered users through education.

Disclosure of interest

The authors declare that they have no competing interest.

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Toxicologie Analytique & Clinique (2017) **29**, 101—110

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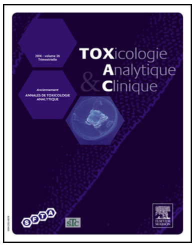

CASE REPORT

Case reports: Four concomitant non-fatal intoxications with AB-FUBINACA and MDMA

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Received 27 October 2016; received in revised form 20 December 2016; accepted 20 December 2016
Available online 19 January 2017

KEYWORDS
Intoxication;
Ecstasy;
Synthetic cannabinoid;
AB-FUBINACA;
Metabolites

Summary We report four cases of non-lethal intoxication with a mix of synthetic cannabinoid (AB-FUBINACA) and ecstasy. Four young (19- to 24-year-old) men were simultaneously admitted to the emergency department after sniffing an unknown white powder in addition of ecstasy consumption. One of them stayed in deep coma for one day, while the three others exhibited tachycardia, hallucinations and dizziness. Toxicological investigations (using immunochemical, LC-DAD, LC-MS/MS and GC-MS methods) revealed the presence of MDMA, MDA and amphetamine in urine and plasma samples collected on admission. A peak related to AB-FUBINACA (ABF) was detected by GC-MS screening and subsequently confirmed by LC-HRMS analysis. Plasma and urine ABF concentrations (determined using LC-SM/SM method) ranged from 1.6 to 44.3 μg/L and from 0.7 to 3.9 μg/L, respectively. Three ABF metabolites were identified using LC-HRMS in plasma and/or urine: an ABF carboxylic acid, a hydroxy-ABF and a hydroxy-desalkyl-ABF. This

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http://dx.doi.org/10.1016/j.toxac.2016.12.006
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report emphasizes the need to investigate synthetic cannabinoids in intoxication cases of young patients.

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Introduction

Since the early 2000s, synthetic cannabinoids (SCs), worldwide abused owing to their psychoactive effects, are the most commonly identified new psychoactive substances (NPS) [1].

SCs present a large variety of chemical structures. There is no clear specific toxidrome related to SC use/abuse out of the context of a clear history of SCs product use [2,3] as symptoms related to SC intoxication are also commonly observed with many other classes of recreational stimulant or hallucinogenic drugs. In 2014, 1.7% of the French general population aged 18—74 claimed to have already smoked a SC. SC users are predominantly men and aged under 35. About 53% of French SC users have already experimented it with at least one illegal substance other than cannabis and 34% have used it with at least two substances [4—6]. In addition to the challenge of identifying SC intoxication, there is a lack of fast analytical methods to confirm SC exposure. Indeed, SCs are usually not detected by routine urinary immunoassays for drugs of abuse, and analytical methods able to detect SCs are available only in research laboratories [7—10]. Castaneto et al. reviewed in 2015 literature published concerning SCs pharmacokinetics and analytical methods for their detection and quantification in biological matrices [11]. Mass spectrometry coupled to gas or liquid chromatography is the golden standard method to analyze SCs.

AB-FUBINACA (ABF), N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carbox-amide, is an indazole SC regularly identified in drug seizures around the word. Pfizer first synthesized it in 2009 as a potent CB1 receptor modulator for potential therapeutic use [12]. It was first detected in Japan in 2012 in illegal herbal products purchased via Internet [13]. However, very few articles reported the presence of ABF in biological samples. The aim of this manuscript is to report four concomitant cases of ABF intoxication.

Case reports

In February 2016, four patients were admitted for recreational drug intoxication to the Emergency Department of Grenoble University Hospital at 7:00 am after a rave party. These young males (patients #1 to 4) were 24, 19, 21 and 23 year-old respectively, without comorbidities or medical history. Patient #2 claimed to the clinicians that they had consumed alcohol (around ten pints of beer), ecstasy (3 or 4 pills), amphetamine and cannabis. They also had consumed by sniff, 2 or 3 times in the evening, an unknown white powder and symptoms started just after this sniffing.

Patient #1 immediately exhibited altered consciousness including acute excited *delirium* and hypotonia. He was directly transferred to Intensive Care Unit in Grenoble Hospital. On hospital admission, he was in deep coma [Glasgow Coma Scale (GCS) estimated to 3], with a nonreactive bilateral mydriasis, hemodynamically stable with no ECG disturbance and he presented hypothermia at 34.5 °C. Intubation was decided over neurological criteria and he was sedated (sulfentanil and midazolam). The brain scanner was normal, hand radiography revealed a fractured metacarpal bone of his right hand. Biological results showed acidosis (pH = 7.28), hyperlactatemia (3.7 mmol/L) and a mild rhabdomyolysis (CPK = 769 UI/L) associated to a minor troponin rise (0.09 μg/L). After extubation (24 hours after the admission) he showed a full consciousness and a normal neurological examination. During the hospitalization, pulmonary radiography showed a pneumopathy successfully treated by amoxicillin-clavulanic acid (IV then per os). He returned home 48 hours after his admission with an addictology follow-up.

Patient #2 experienced visual hallucinations (color deformation) and attention disorders with impaired alertness for about 2 hours. At the Emergency department, he presented a reactive bilateral mydriasis with multidirectional nystagmus and was not stable at Romberg balance test. The remaining neurological examination was normal (GCS 15). His heart rate was 130 bpm at admission. Biological results revealed no abnormality and he returned home 12 hours after the admission.

Patient #3 vomited before the first aid arrived. On hospital admission, he had nonreactive bilateral mydriasis with drowsiness and dizziness, and he experienced intense episodes of pallor and sweetness. His heart rate was 112 bpm. He described blurred vision and seemed anxious. He stayed asthenic all day long while pallor regressed (GCS 14—15). He had an addiction checkup 24 hours after the admission and returned home.

Patient #4 experienced visual hallucinations and had an anxiety attack. At the Emergency department, he had a nonreactive bilateral mydriasis with dizziness (GCS 14—15). His heart rate was 114 bpm and he experienced urinary retention. He remained drowsy all day long and went back home 24 hours after the admission.

Material and methods

For all patients, plasma and urinary samples were handled on admission. The samples were stored at 4 °C until analyses.

Chemicals

Methanol (MeOH) (LiChrosolv), acetonitrile (CH_3CN) (Pestipure), ethyl acetate (HiPerSolv), ammonia solution 25%, were purchased from Merck (Darmstadt, Germany). Formic acid (analytical grade) was provided by Prolabo (Paris, France). Ammonium formate was purchased from Sigma-Aldrich (St Quantin-Fallavier, France). Sulfosalicylic acid was provided by Carlo Erba (Val-de-Reuil, France). Ultra-pure water (resistivity $\geq 18.0\text{M-}\Omega/\text{cm}$) was generated using a Milli-Q Plus (Millipore, Molsheim, France). Toxivial A® extraction devices were purchased from Interchim (Montluçon, France). N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane, Trimethylsilyl chloride (BSTFA 1% TMCS), β-glucuronidase from Helix Pomatia, and ABF were purchased from Sigma-Aldrich (St Quentin-Fallavier, France).

Immunochemical screenings

Immunoassays of the 4 urines samples were performed using Vista analyzer (system dimension Vista, Siemens Healthcare Diagnostics Inc, Siemens). Syva® EMIT® II Plus AMPH Flex®, Syva® EMIT® II Plus EXTC Flex®, Syva® EMIT® II Plus COC Flex®, Syva® EMIT® II Plus OPI Flex®, Syva® EMIT® II Plus THC Flex® (Flex Reagent Cartridge; Siemens Diagnostics) were respectively used to search amphetamines, ecstasy, cocaine, opiates and cannabis. Buprenorphine was tested in urines by immunochromatography (TOX/See™, BIO-RAD). Benzodiazepines and ethanol were tested in plasma and urine using Syva® EMIT® toxTM kit and ETOH kit (Flex Reagent Cartridge; Siemens Diagnostics), respectively.

Gas chromatography-mass spectrometry analysis

Assay was performed using an Agilent Technologies 6890N Network GC System combined with an Agilent Technologies 5975 network mass selective detector and an Agilent Technologies 7683 series injector. The entire process, including data collection, was controlled using the Agilent Technologies MSD ChemStation E.02.02.143.

Sample [0.5 mL of overnight hydrolyzed (using 60 μL of 25,000 U/mL helix pomatia glucuronidase solution) and 0.5 mL of non-hydrolyzed urine] preparation consists of a liquid extraction Toxivial A® after internal standard (SKF-525A) addition [14].

The extract was reconstituted with 60 μL of ethyl acetate (AE) and separated in two subsequent extracts, one was kept in AE, the other was derivatizated using BSTFA 1% TMCS (1 h, 65 °C). 1 μL of each extracted sample was injected in a DB-5ms UI column (30 × 0.25 mm internal diameter; 0.25 μm film thickness) using pulsed splitless injection with an injector temperature of 250 °C. Temperature conditions were as follows: initial temperature of 70 °C for 1 min, increased to 100 °C at 10 °C/min, then increased to 300 °C at 20 °C/min and held for 12 min. The flow of the carrier gas (helium) was maintained at 1 mL/min in constant flow mode and the gas chromatograph interface temperature was held at 315 °C. Electron impact ionization was performed at 70 eV, with an ion source temperature of 230 °C and mass spectra collected in scan mode (from 40 to 600 m/z). Obtained mass spectra (EI) were evaluated against NIST11, PMW2007 and SWGDRUG libraries [15].

Liquid chromatography-tandem mass spectrometry method for amphetamine determination

The CLHP system consisted of 3 pumps LC 20AD (Shimadzu LC Prominence) equipped with an autosampler (SIL 20 AC) and a mass spectrometer detector AB SCIEX 3200 QTRAP (ABSciex Toronto, Ontario, Canada) equipped with an electrospray ESI probe (positive mode). Sample preparation consists of 200 μL of plasma or urine treated with 200 μL of sulfosalicylic acid (500 mg/mL) containing ISs. Fifty microliters of supernatant were injected in the chromatographic system [16]. The on-line sample clean-up was performed using an Oasis HLB column (2.1 mm × 20 mm; 25 μm, WATERS) and the chromatographic separation was performed on an analytical column, X Select® HSS T3 (100 × 2.1 mm; 2.5 μm, WATERS) thermostated at 45 °C. The detection was operated in positive mode under the following conditions: positive ESI electrospray voltage: 5500 V; nebulization gas flow rate: 50 psi; turbo heater gas flow rate: 60 psi; ion source temperature: 650 °C. The analyses were performed in multiple reaction monitoring (MRM) mode. The monitored transitions (collision energies) were 136.2/119.2, 91.1 (11/27) for amphetamine, 150.2/91.1, 119.2 (23/13) for metamphetamine, 180.2/135.2, 105.2 (23/25) for MDA and 194.2/163.3, 105.2 (15/33) for MDMA.

Liquid chromatography-diode array detection analysis

Liquid chromatography-diode array detection analysis was described by Reynaud et al. [17].

Liquid chromatography with high-resolution mass detection and tandem mass spectrometry detection methods for ABF (and metabolites) identification and determination

In agreement to the literature recommendations, ABF and its metabolites were subsequently identified in plasma and urine using a previously reported LC-HMRS method [18—20]. The Waters ultra-performance liquid chromatography system consisted of two binary solvent manager LC pumps, a sample manager autosampler and a column manager oven Acquity. Mass spectrometry data were acquired using a XEVO G2-XS QTOF (Waters) instrument controlled with MassLynx 4.1 software. Extractions of samples (75 μL of plasma or urine) were performed after addition of internal standards (methyl-clonazepam and β-OH-ethyltheophyllin) using an OASIS HLB on-line column (30 × 2.1 mm, 20 μm) (Waters). The chromatographic separation was performed using an ACQUITY HSS C18 column (150 × 2 mm, 1.8 μm, Waters) in an oven temperature of 50 °C, and mobile phases including ammonium formate buffer 5 mM, pH 3 (A) and acetonitrile in 1% formic acid (B). For detection, mass spectrometric conditions were as follows: positive electrospray ionization interface (ESI+), ion spray voltage set

Table 1 Analytical results in urine and plasma samples of the 4 patients.

	MDMA (μg/L)	MDA (μg/L)	Amphetamine (μg/L)	THCCOOH	ABF (μg/L)	Metabolites of ABF	Others
Patient #1							
Plasma	544	25.7	5.1		18	ABF carboxylic acid Hydroxy-ABF Desalkyl-ABF	
Urine	39,200	1112	< 5	Negative	3.9	ABF carboxylic acid Hydroxy-ABF Desalkyl-ABF	Propofol, lidocaine
Patient #2							
Plasma	420	39	< 5		1.6	ABF carboxylic acid Hydroxy-ABF Desalkyl-ABF	
Urine	8440	428	5.0	Positive	0.7	ABF carboxylic acid Hydroxy-ABF	
Patient #3							
Plasma	300	14.3	16.6		44.3	ABF carboxylic acid Hydroxy-ABF Desalkyl-ABF	
Urine	26,560	1116	1160	Positive	1.8	ABF carboxylic acid	
Patient #4							
Plasma	816	26.3	< 5		30.2	ABF carboxylic acid Hydroxy-ABF Desalkyl-ABF	
Urine	22,840	716	9.5	Negative	0.7	ABF carboxylic acid Hydroxy-ABF Desalkyl-ABF	Acetaminophen

THCCOOH: 11-nor-9-carboxy-Δ^9-THC; ABF: AB-FUBINACA.

at 20 V, source temperature set at 140 °C and desolvation temperature at 500 °C with a desolvation gas flow rate of 900 L/h, nitrogen as desolvation gas and argon as collision gas. Data process was performed using ChromaLynx, TargetLynx, MassFragment and MetaboLynx associated softwares (Waters) using a homemade database of more than 1400 substances including nearly 300 NPS (including ABF) or metabolites.

ABF concentrations were subsequently determined in plasma and urine samples using a LC-MS/MS method (XEVO TQ-S system, Waters) as follows: 100 μL of plasma or urine sample was deproteneinized (300 μL methanol), added with internal standard (100 μL of methanol-containing β-OH-ethyltheophyllin at 1.6 μg/L), diluted (200 μL of ammonium formate/0.1% formic acid buffer), and analyzed after separation by an Acquity UPLC HSS C18 column (1.8 μm, 2.1 × 150 mm, Waters) using a gradient of (A) 5 mM ammonium formate/0.1% formic acid buffer, and (B) ACN/0.1% formic acid as mobile phase at a flow rate of 0.4 mL/min during 5 min. Xevo TQ-S tandem mass spectrometer was used for detection after positive electrospray ionization mode in the MRM mode using the following transitions: m/z 369.1 to 253.1 (for quantitation) and m/z 369.1 to 324.1 for ABF; m/z 225.1 to 124.1 (for quantitation) and 225.1 to 81.1 for IS (β-OH-ethyltheophyllin).

Results

Similar toxicological results were obtained for the four patients: plasma ethanol levels were below 0.1 g/L. Qualitative urine immunoassays were negative except for amphetamine and ecstasy assays and for cannabis assay for two patients. These positive results for amphetamine and ecstasy were confirmed and quantified by the LC-MS/MS method (see Table 1) that also excluded the presence of metamphetamine, MDEA, MBDB or mephedrone.

Screening using LC-DAD showed the presence of lidocaine and propofol in plasma and urines of the patient 1, MDMA for the 4 patients. In addition to these compounds the GC-MS screening allowed to detect acetaminophen in urine of the patient 4, and 11-nor-9-carboxy-Δ^9-THC (THCCOOH) in urine of patients 2 and 3.

This GC-MS screening also revealed in all urine samples the presence of an unknown substance (retention time of 16.36 min) (previously derivatizated with BSTFA 1% TMCS). The mass spectrum (see Fig. 1) corresponded to ABF after comparison with SWGDRUG library (Agilent format). However, no peak was obtained at 16.36 min when ABF reference standard was injected. As shown on Fig. 2, two peaks (at 17.21 and 17.90 min) were identified as ABF, when ABF reference standard was treated as patient samples (hydrolyzed using β-glucuronidase and derivatizated

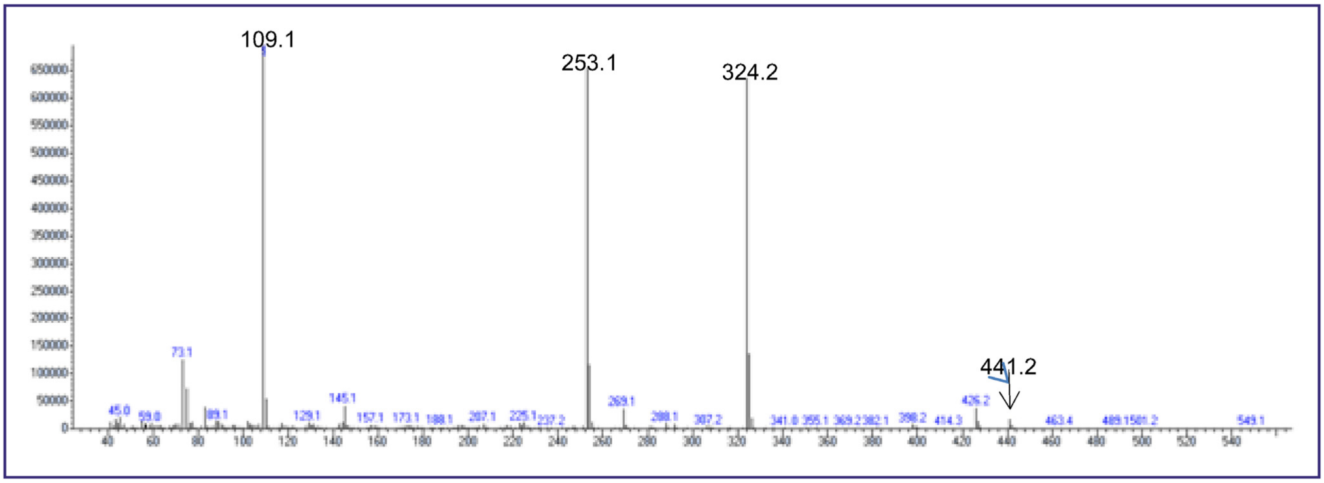


Figure 1. GC-MS mass spectrum observed at 16.36 min of urine extract of patient #3 (hydrolyzed with β-glucuronidase and derivatizated with BSTFA 1%TMCS).

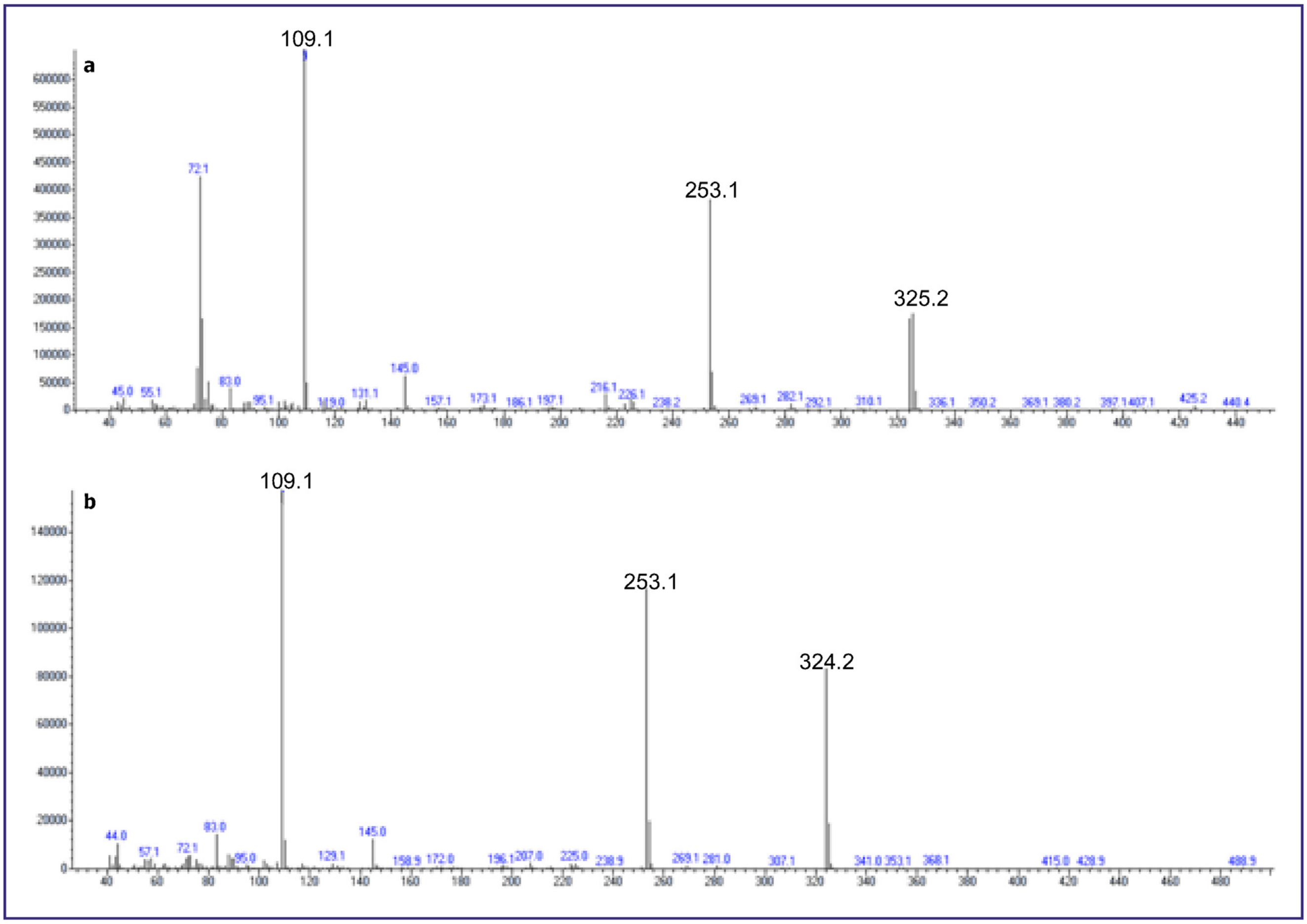
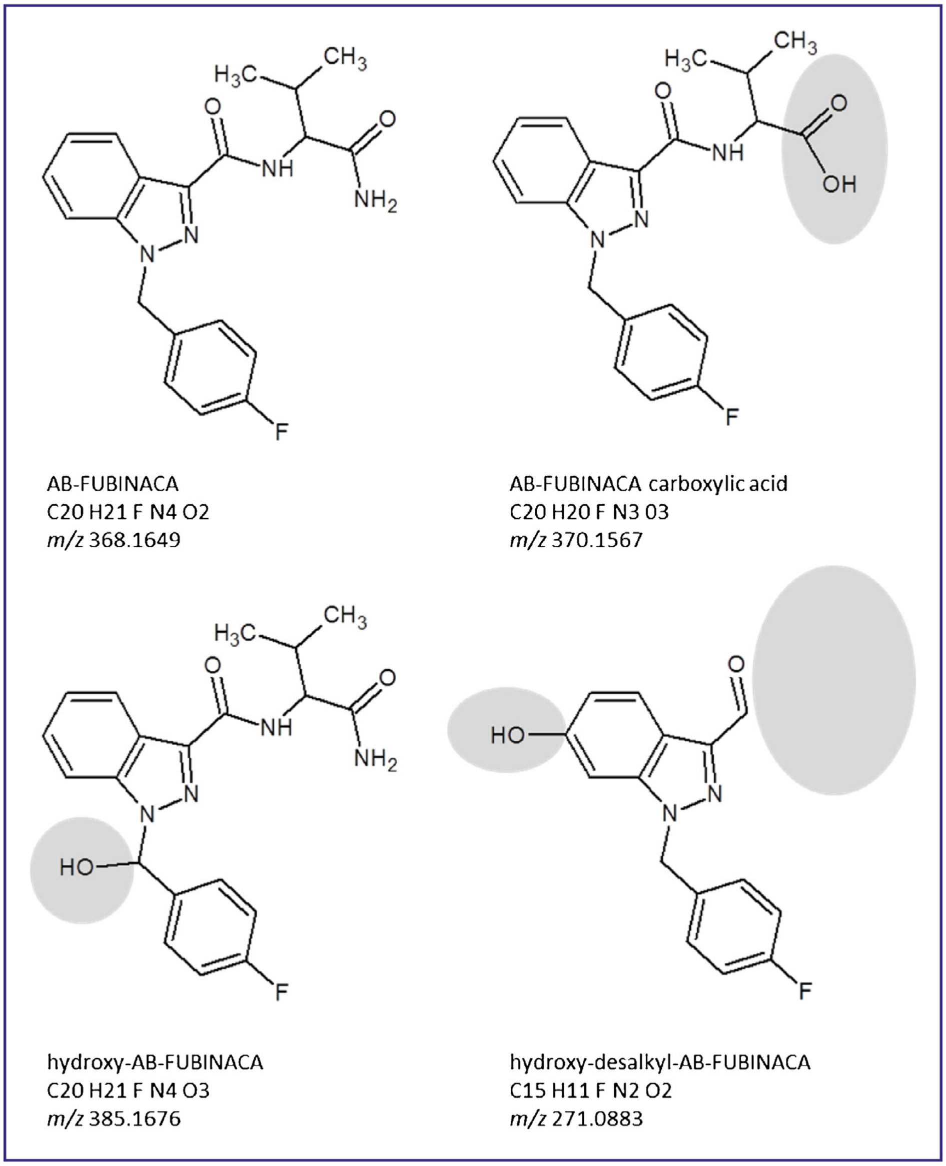


Figure 2. a: GC-MS mass spectrum observed at 17.21 min for AB-FUBINACA standard sample hydrolyzed with β-glucuronidase and derivatizated with BSTFA 1%TMCS; b: GC-MS mass spectrum observed at 17.90 min for AB-FUBINACA standard sample hydrolyzed with β-glucuronidase and derivatizated with BSTFA 1%TMCS.

with BSTFA 1% TMCS). It could be noticed that one peak was mainly observed at 17.90 min, when ABF reference standard was not hydrolysed using β-glucuronidase. As a consequence, biological samples were sent to a reference laboratory for confirmation (and quantitation) of ABF. Using LC-HRMS method, ABF presence was confirmed in urine and in plama (ABF + H^+, *m*/*z* 369.1727, retention time of 9.76 min), and 3 ABF metabolites were identified: a ABF carboxylic acid (amide hydrolysis) (*m*/*z* 370.1567, retention time of 10.28 min), a hydroxy-ABF (*m*/*z* 385.1676,



AB-FUBINACA
C20 H21 F N4 O2
m/z 368.1649

AB-FUBINACA carboxylic acid
C20 H20 F N3 03
m/z 370.1567

hydroxy-AB-FUBINACA
C20 H21 F N4 O3
m/z 385.1676

hydroxy-desalkyl-AB-FUBINACA
C15 H11 F N2 O2
m/z 271.0883

Figure 3. AB-FUBINACA and observed metabolites.

retention time of 8.35 min) and a hydroxy-desalkyl-ABF (internal amide hydrolysis and hydroxylation with indazole intact) (*m/z* 271.0883, retention time of 10.28 min) (Table 1, Figs. 3 and 4). Considering relative peak area surfaces, the ABF carboxylic acid was the most abundant metabolite in all samples (plasma or urine). For the hydroxy-ABF metabolite, considering fragmentation, hydroxylation on the carbon atom close to the fluorobenzyl group is the more probable hypothesis. Nevertheless, possibility of hydroxylation on the amino-oxobutane moiety or on the indazole ring can be considered. The exact hydroxylation site of the hydroxylated metabolite remains uncertain and further investigation is needed to define the exact structure.

Plasma and urinary ABF concentrations subsequently determined using a LC-MS/MS method [LLOQ: 0.5 μg/L, ULOQ: 100 μg/L, interday CV and bias ($n = 5$) < 20% in plasma and in urine] ranged from 1.6 to 44.3 μg/L and from 0.7 to 3.9 μg/L, respectively (Fig. 5).

Discussion

Toxicological investigations revealed the presence of MDMA, MDA, amphetamine and ABF and its metabolites in urines from the four patients. Patients #1 and #4 had plasma MDMA concentrations over 500 μg/L, which is considered as toxic [21,22] (Table 1). Since amphetamine plasma concentrations are very low, a consumption of amphetamine (as ''speed'' pills) cannot be excluded. However, the positive result for amphetamine is likely to be related to the intake of ecstasy pill in which amphetamine small amount can be present as synthesis impurity. Intoxication with NPS is not easy to diagnose, especially when used in combinations with other drugs, as seen in these 4 cases. Here, the concomitant intake of MDMA did not allowed clinicians to draw a clear toxidrome related to ABF consumption [23]. Thus, the hospitalization can be related to sniff of ABF white powder in addition to massive consumption of MDMA.

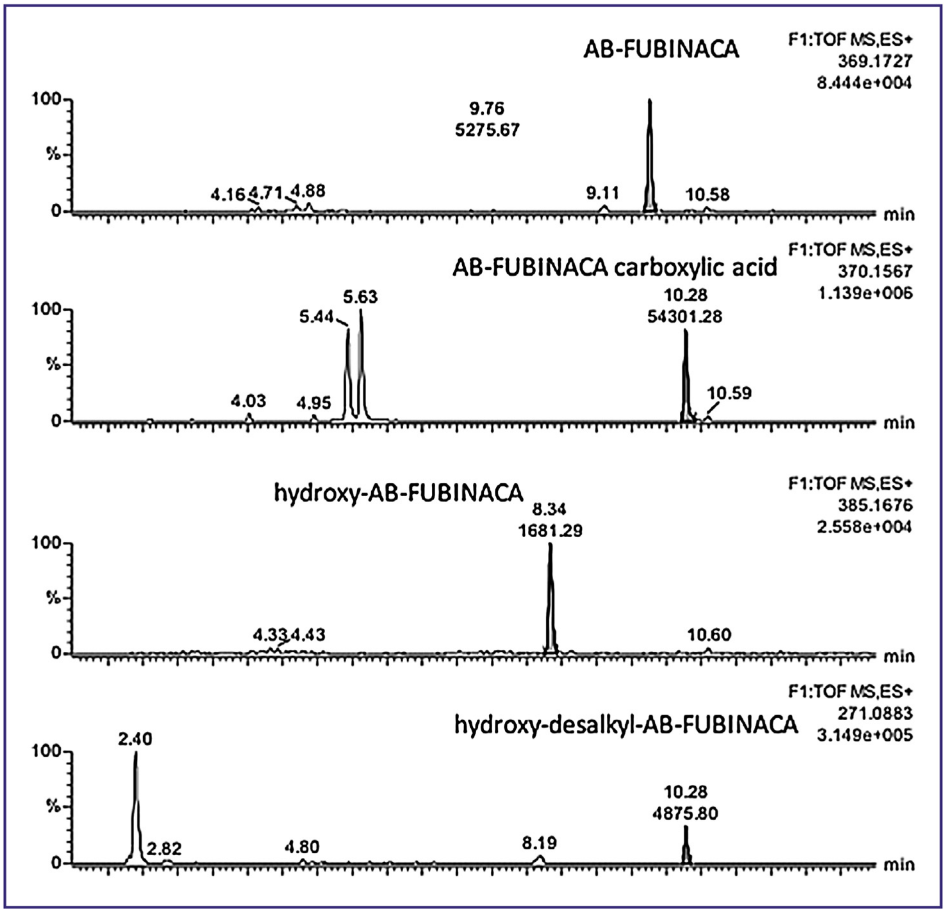


Figure 4. LC-HRMS chromatogram of AB-FUBINACA and metabolites in serum of patient #4.

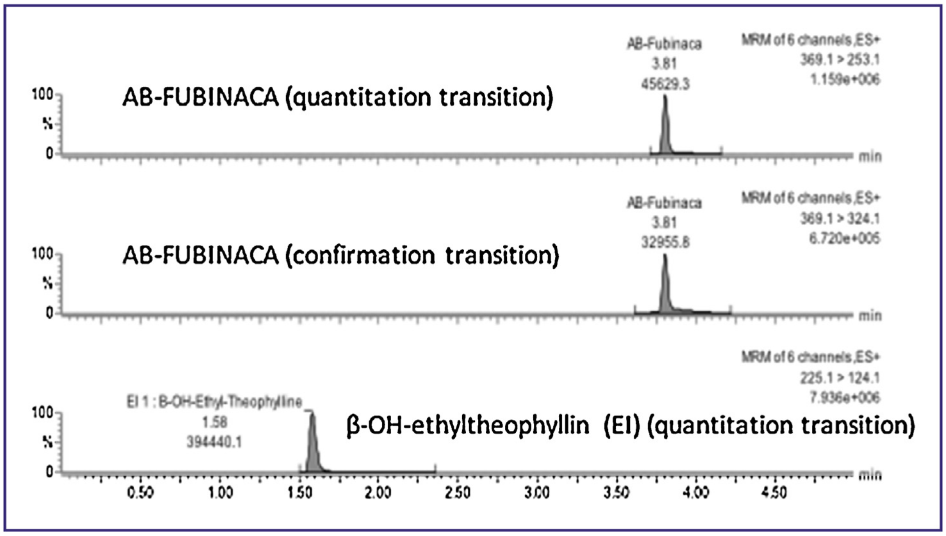


Figure 5. LC-MS/MS chromatogram of AB-FUBINACA in serum of patient #4; AB-FUBINACA concentration is 30.2 μg/L.

The most common route of ABF administration is inhalation (via water pipe/bong, cigarette, blunt or pipe) although oral and rectal administration routes have also been mentioned. Sniffing pure SC powder is an unusual way since it can lead to severe overdose by underestimating the dosage [24].

In routine practice, simple screening methods are required for the detection of SCs in seized or biological materials. SCs can be identified in blood, oral fluid, and hair, whereas metabolites are predominantly identified in urine with a larger window of detection. At the moment, there are no commercial assays for the routine screening

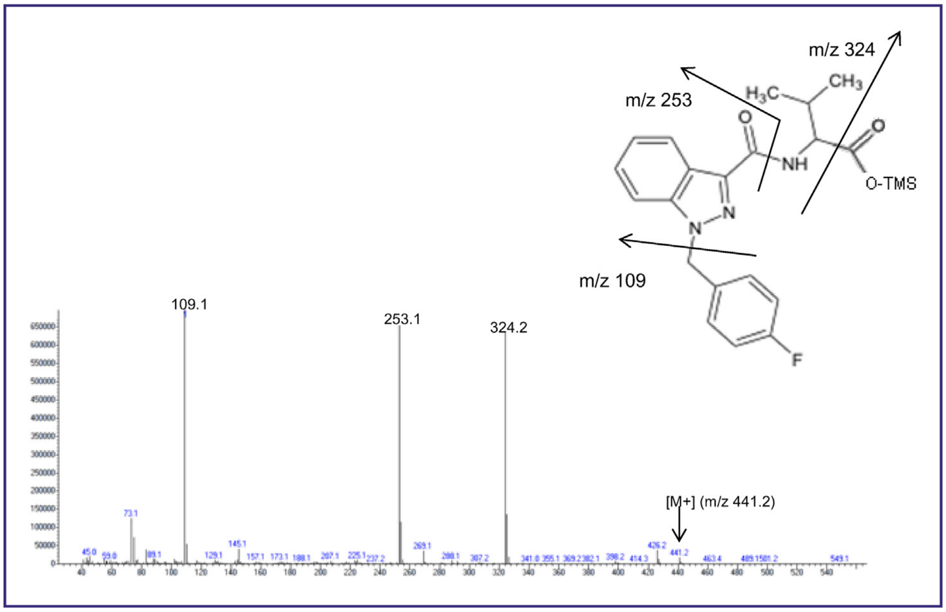


Figure 6. Derivatizated AB-FUBINACA (ABF) carboxylic acid possible fragmentation.

of these drugs in biological samples [8]. In these 4 cases, a GC-MS screening method was performed as the first-line investigation. The urinary GC-MS screening identified a peak at 16.36 min as ABF by comparison with SWGDRUG library (Fig. 1). But the injection of ABF reference standard showed two peaks at different retention times (Fig. 2). Thus, in urinary samples the compound identified as ABF by GC-MS was probably a urinary metabolite of ABF i.e. ABF carboxylic acid as suggested by Fig. 6.

Identification and quantification of SCs and their metabolites are crucial to document their use and try to correlate their toxicity to their concentrations. Few articles have reported the presence of ABF in human biological samples. A case of intoxication with a mix of psychoactive substances including ABF has been described [25]. In this report, ABF was detected by GC-MS in drugs purchased via Internet and in the stomach content of the patient. However, the authors failed to identify ABF in urine. In France, SCs are not the most common seized NPS [26]. Nevertheless, ABF has been identified in seized products in 2014 by forensic toxicology laboratories of the French Police Forces and more recently in dry herbs in southeast France [26,27]. Recently, Vaiano et al. reported the case of a 15-year-old girl brought to the local hospital after collapsing twice at school [28]. She declared to have smoked a ''synthetic herb'' with her friends before going to class: ABF blood concentration was 4.6 μg/L, in agreement with plasma concentrations range (1.6 to 44.3 μg/L) observed in the present paper. Detection in urine of parent compound of SCs is unusual. For example, ABF was detected in urine in only 54% (15/28) of ABF intake cases in a recent study [29]. Thus, the relative low urinary concentrations versus plasma concentrations observed in our 4 cases are consistent with previous data.

The metabolism of ABF has been studied using human hepatocytes or human liver microsomes (HLM) [11,29]. The oxidation of ABF leading to a hydroxylated metabolite has been reported to occur on the N-(1-amino-alkyl-1-oxobutan) moiety [30]. The major metabolic pathways that have been determined consist in terminal amide hydrolysis leading to ABF carboxylic acid, acyl glucuronidation and hydroxylation on the amino-oxobutane moiety, while epoxidation followed by hydrolysis, hydroxylation at the indazole moiety and dehydrogenation were minor pathways (defluorination did not occur.). ABF carboxylic acid, hydroxy-ABF carboxylic acid and ABF carboxylic acid glucuronide were the three most prevalent metabolites in authentic urine specimens [31]. In a very nice study based on authentic case samples together with HLM incubations, Vikingsson et al. recently established that three metabolites (M9, M13, and M18) could be urinary biomarkers of an ABF intake: M9 was the result of a monohydroxylation on the indazole ring, M13 a monohydroxylation on the amino-oxobutane moiety, and M18 a hydrolysis of the primary amide (ABF carboxylic acid) [29]. In the 4 reported cases, the ABF carboxylic acid metabolite was the main observed metabolite, in agreement with these 2 later studies. The presence of a hydroxyl-ABF metabolite (even if the exact position of the hydroxylation remains uncertain) was confirmed. In contrast, we observed the presence of a hydroxy-desalkyl-ABF (internal amide hydrolysis and hydroxylation with indazole intact), which has not been previously reported as a metabolite of ABF. These data suggested a great inter-individual variability of ABF metabolism and should be confirmed in further studies.

Conclusion

To our knowledge, this is the first report of intoxication cases documented by the identification of ABF and its metabolites in plasma and urine. For one patient (patient #1), sniffing ABF powder and MDMA consumption has led to severe intoxication requiring intensive care support. These 4 cases highlight the need for toxicological laboratories to include SCs in their screening procedures in order to reduce risks associated with acute intoxication in emergency context or chronic use in addiction context. Here, this approach consisted of a GC-MS screening [14] able to detect an ABF derivative in urine samples, and of LC-HRMS confirmation [32] in a second step.

Disclosure of interest

The authors declare that they have no competing interest.

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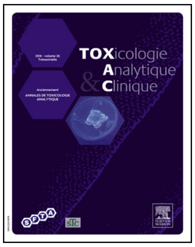

CASE REPORT

Madness with five dollars: Two new cases of non-lethal poisoning flakka (α-PVP)

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Received 6 November 2016; received in revised form 28 November 2016; accepted 28 November 2016
Available online 4 January 2017

KEYWORDS
α-PVP;
New psychoactive substances;
Flakka;
Synthetic cathinones;
Hair analysis

Summary We reported the case of two young men, known to be polydrug abusers, arrested in possession of plastic bags of crystals and capsules (total about 300 grams) suspected to be narcotics or new psychoactive substances (NPS). Placed in custody, they will quickly hospitalize for their bad condition (tachycardia, dysarthria, great excitement) where blood samples are taken. Hair samples will be taken for the two subjects, respectively, 1 month and 4 months after hospitalization. Analyses of seized materials, blood and hair samples were requested by the justice and sent to the laboratory for identification and document the drug exposure. Identification of seized materials was performed with gas chromatography-mass spectrometry (GC-MS) using commercial and free specialized mass spectra libraries. Blood toxicological analysis was performed by gas chromatography with flame ionization detection (GC-FID), high performance liquid chromatography-diode array detection (HPLC-DAD), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS/MS). Several hair segments were analyzed by LC-MS/MS. Alpha-pyrrolidinopentiophenone or alpha-pyrrolidinovalerophenone (α-PVP, also known as street name ''flakka'') and pyrrolidinoheptiophenone (PV8, also known as α-PHpP or α-PEP) were identified in seized materials. Toxicological analysis in peripheral blood revealed the presence of α-PVP (100 and 70 ng/mL, respectively), 2-oxo-PVP (50.4 and 22.7 ng/mL, respectively), PV8, diazepam and metabolites for the two subjects. Hair analysis demonstrated the presence of α-PVP (0.88 and 0.19 to 1.99 ng/mg, respectively), 2-oxo-PVP, morphine, cocaine and metabolites, amphetamine, MDMA, methadone and EDDP and suggested a repeated consumption of these substances.

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http://dx.doi.org/10.1016/j.toxac.2016.11.006
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Introduction

These last years, new psychoactive substances (NPS) have invaded the drug market. They are designed to cause similar effects to drugs of abuse and are considered as ''legal'' alternative for some of them, hence the name of legal high and purchasing is very easy on specialized websites. In France, the synthetic cathinone chemical family is the most represented in drug seizures in the years 2014 and 2015. Alpha-pyrrolidinopentiophenone (alpha-pyrrolidinovalerophenone or α-PVP) has been available in the European Union (EU) since at least February 2011 and has been detected in 28 Member States, Turkey and Norway. In most cases it has been seized as a powder, but other forms including tablets have been detected. Multi-kilogram quantities of α-PVP have been seized at European borders, which usually originate from China. This includes the seizure of more than 280 kg in 2015 [1]. Illicit production and tableting sites within the EU have also been seized [1]. The substance α-PVP is sold online as a ''research chemical'' but also as ''bath salts'', ''plant food'' or ''insect repellers'' and was seized for the first time in France in 2011 as the PV8 (α-PHpP, α-PEP or pyrrolidinoheptiophenone) was seized in 2014 [2]. The substance α-PVP is a stimulant drug chemically related to MDPV (methylenedioxypyrovalerone). Both are pyrovalerones. Pharmacological description and health risks are described in the literature [3,4]. User reports indicate that it can be swallowed, insufflated, administered sublingually, and vaporized [1,3,4]. Many cases of intoxication, violent aggressive behavior and madness attacks were described in 2015 in the USA (Florida), widely reported in the international press. Presence of α-PVP is reported in polydrug overdoses and sometimes where the drug was found to be a significant contributory cause to the death. A total of 116 deaths associated with α-PVP (including 2 in France) were reported in Europe between 2012 and 2015 and 205 acute intoxications (including 10 in France) were reported in Europe in the same period [3]. Much of the synthetic cathinones are classified as drug of abuse in France since the decree of 27 July 2012 with for the first time, a new approach called ''generic'' classification with a single text [5].

Despite increasing use of such compounds in France, only few intoxications or fatalities concerning flakka have been reported with complete analytical data.

This case report documents two severe intoxications with this synthetic cathinone and the concentrations measured in blood and hair.

Case report

In December 2015, a seizure of crystals and capsules containing a white powder is performed on 2 men, who are placed in custody at the police station for drug trafficking.

During the interrogation, one of them says he had consumed products called α-PVP and described the effects usually felt by injection:

> *''After the injection, I have a flash for about 10 minutes, after I feel buzzy for 12 hours then it starts to feel like black holes''.*

Nevertheless, quickly after the arrest, they started to have disturbing clinical signs before their transfer to emergencies.

The subject 1 is a 27-year old man addicted on multiple drugs (alcohols and many psychoactive substances) which readily admits taking α-PVP. He indicates having injected 10 mg of α-PVP several times for 4 days having smoked it for 3 weeks. In the police station, he presents discomfort with short clonies and dysarthria. He indicates that he has self-injected 10 mg of α-PVP several times for 4 days having smoked it for 3 weeks. On admission to the emergency department, he presents tachycardia, is normotensive, and has no fever. He has no disturbance of consciousness but dyskinesias and a major agitation. The CPK value is increased to 1800 IU/L (N: 5—170). He is treated symptomatically by Valium, Skenan, Tercian, Lepticur and then transferred to a psychiatric hospital for the rest of the treatment.

The subject 2 is a 34-year old man, also addicted to multiple drugs (cannabis, amphetamines, Valium and Skenan); during his custody, he said he had not taken any drugs in the last 12 hours, yet his state of consciousness gets progressively worse and he was transferred for sleepiness to the emergency department. At the admission, the Coma Glasgow Scale is 14; he has tachycardia, and is normotensive. He is hospitalized for a neurological monitoring. Clinical signs improve spontaneously without treatment other than perfusion. Then, he is transferred in infectious diseases department for the treatment of an abscess at the injection site.

For these 2 patients, blood toxicology samples were collected on admission to the emergency department. Capillary samples will be later made for analysis: one month later (3 cm) for case 1 and four months later (20 cm) for case 2. The powder contained in capsules and bags seized during the arrest were also submitted for analysis.

Material and methods

Seized materials analysis

A sample of the contents of each bag: capsule contents for the bag 1 (Fig. 1) and 20 mg of crystals for the bags 2 and 3 (Figs. 2 and 3) were dissolved in methanol. The analyses were performed using a gas chromatograph (HP 6890 GC System, Agilent Technologies, USA) coupled to a mass spectrometer (quadruple mass analyzer Agilent 5973, Agilent Technologies, USA). The injector was maintained at 280 °C. Sample injection (1 μL) was in splitless mode. Components separation was conducted on a HP-5MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness, Agilent Technologies USA). Helium was used as a carrier gas. The mass detector was set to positive electron impact mode (EI) and the electron beam energy was 70 eV. The mass detector was operating in a full scan mode in the range of 29—600 amu. The acquisition and results analysis were conducted with MSD ChemStation (Agilent Technologies, USA). Identification of substances was supported by comparison of its mass spectrum with spectra of standards included in commercial and free specialized libraries (e.g. SWGDRUG Mass Spectral Library version 2.3 and Cayman Spectral Library [CSL]) [6,7].

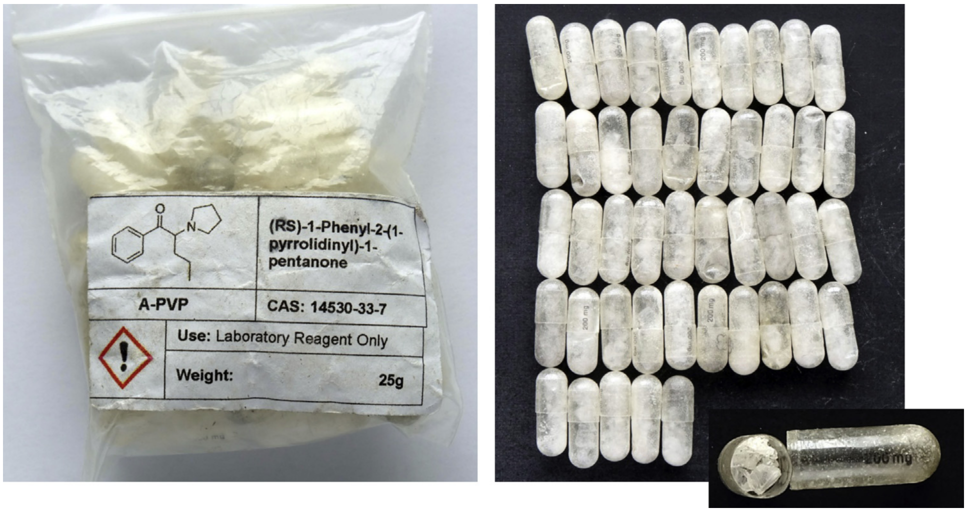
(RS)-1-Phenyl-2-(1-pyrrolidinyl)-1-pentanone
A-PVP
CAS: 14530-33-7
Use: Laboratory Reagent Only
Weight: 25g
200 mg

Figure 1. Seized materials — bag 1 containing capsules.

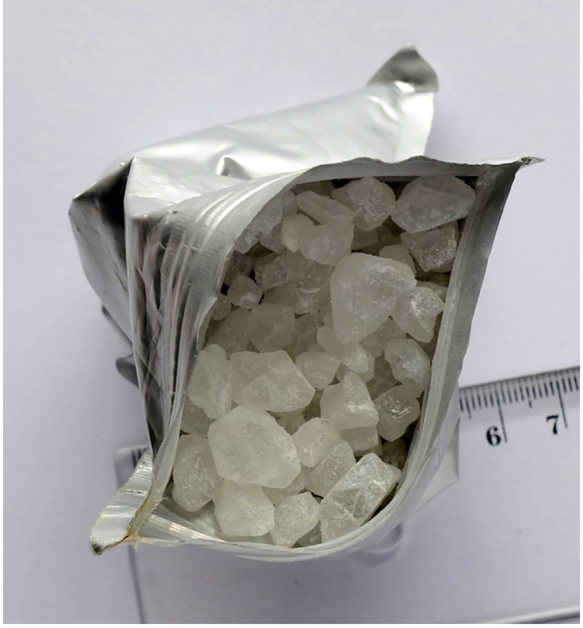

Figure 2. Seized materials — bag 2 containing translucent crystals.

Blood analysis

Peripheral blood of the two subjects was screened for alcohol, drugs of abuse and pharmaceuticals by routine procedures including immunoassays, headspace gas chromatography with flame ionization detection (HS-GC-FID), liquid chromatography coupled with a diode array detector (HPLC-DAD) and gas chromatography coupled with mass spectrometry (GC-MS).

A routine and specific screening for amphetamines, opiates, cocaine and NPS included α-PVP and the metabolite 2-oxo-PVP was performed on peripheral blood by ultra-performance liquid chromatography-mass tandem spectrometry (2D-UPLC-MS/MS) using a Waters Acquity system with an online solid phase extraction. Samples were extracted using Ostro 96-well sample preparation plate (Waters): 20 μL of blood sample and 20 μL of internal standard (mephedrone-d3 in methanolic solution) were added to wells, 800 μL of an aqueous solution of $ZnSO_4$/acetonitrile/methanol 0.2 M were then added for protein precipitation. The mixture was drawn and discharged several times. Samples are placed in the freezer 10 min and then eluted into a 2 mL 96-well collection plate. Twenty microliters of supernatant were injected in the system using X Bridge C18 column (Waters) as trapping column and Acquity BEH C18 column (Waters) as separation column. Separation was achieved with a gradient delivered at a flow rate of 0.3 mL/min of a mixture of 0.1% formic acid in ACN and 0.1% formic acid in desionized water. A XevoTQD triple-quadrupole mass spectrometer (Waters) was used for detection and ionization was achieved using electrospray in the positive ionization mode (ES+). Two MRM dynamic multiple reaction monitoring transitions (quantification and confirmation) for all compounds of interest were selected and optimized. Transitions *m*/*z* 232.1 to 91 and 232.1 to 105; *m*/*z* 246 to 90.9 and 246 to 160.9 and 181.09 to 148.02

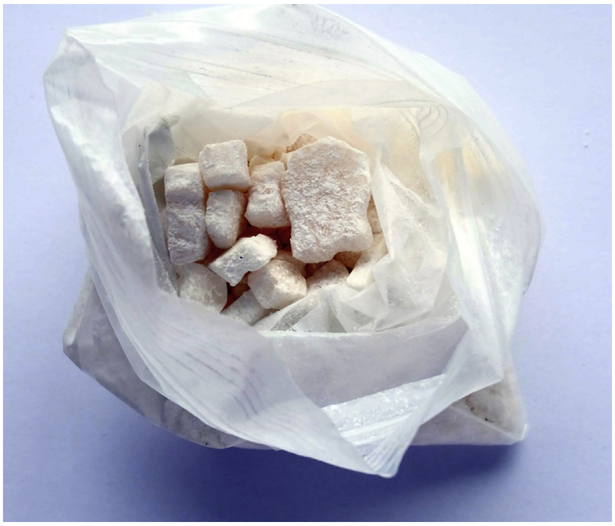

Figure 3. Seized materials — bag 3 containing white crystals.

were used for α-PVP, 2-oxo-PVP and mephedrone-d3 (IS), respectively. Data acquisitions were done using MassLynx 4.1 software (Waters).

Hair analysis

After a twice decontamination with methylene chloride, the hair strand was analyzed without segmentation for subject 1 (one segment, length = 3 cm) and segmented into four segments of four centimeters for subject 2 (The terminal segment was not analyzed). Each segment was cut into small pieces (< 1 mm) and 10 mg were incubated 12 h at 56 °C in a mixture of methanol/acetonitrile/formate buffer 2 mM (25/25/50, v/v/v) with 20 ng of mephedrone-d3 in methanol (500 ng/mL) used as internal standard. Samples were centrifuged and the supernatant was evaporated to dryness under nitrogen at 40 °C. The dry extract was reconstituted in 130 μL of the mobile phase, filtrated, and 50 μL were injected on the 2D-UPLC-MS/MS system (Waters Acquity and XevoTQD triple-quadrupole mass spectrometer). Columns, mobile phase and chromatographic conditions were the same as for the analysis of blood samples.

Results

Seized materials analysis

The analyses revealed in the capsules of the bag 1 and in the crystals of the bag 2, the presence of α-PVP (alpha-pyrrolidinopentiophenone or alpha-pyrrolidinovalerophenone) and PV8 or α-PHpP or α-PEP (alpha-pyrrolidinoheptiophenone) in the crystals of bag 3. Mass spectra are showed in Fig. 4 with major ions $m/z = 126$ and $m/z = 154$ for α-PVP and PV8, respectively. Chemical formula are $C_{15}H_{21}NO$ and $C_{17}H_{25}NO$, molecular weight are 231 g/mol and 259 g/mol for α-PVP and PV8, respectively. The difference between the 2 molecules is found at the level of the length of the longer side chain in PV8.

Blood and hair analysis

The screening of peripheral blood revealed a positive result for benzodiazepines (diazepam and metabolites) and a negative result for alcohol, opiates, cocaine, amphetamines and narcotics, for both subjects. Recent use of cannabis was found in the subject 2.

The 2D LC-MS/MS method was validated before routine use and indicated a limit of detection (LOD) at 0.5 ng/mL, a limit of quantification (LOQ) at 1 ng/mL and a linearity ranging from 1 to 100 ng/mL for α-PVP and 2-oxo-PVP in whole blood. Precision has been checked by inter-day CVs and associated relative bias ($n = 5$), which were lower than 20% for each compound. The limit of quantification for hair was 0.05 ng/mg and was acceptable for hair analysis.

PV8 in blood and hair was not quantified because it was not possible to get the standard.

The 2D LC-MS/MS analysis revealed the presence of α-PVP, 2-oxo-PVP and PV8 in the blood of the 2 subjects. Results are presented in the Table 1. Analysis of hair strand revealed the presence of α-PVP, 2-oxo-PVP, morphine, cocaine, benzoylecgonine, norcocaine, amphetamine, MDMA, methadone and metabolite EDDP for subject 1 and in all the segments tested for subject 2.

Results are presented in Tables 2 and 3.

Table 1 Results of blood analysis of the two subjects (ng/mL).

Compound	Subject 1	Subject 2
α-PVP	100	70
2-oxo-PVP	50.4	22.7
PV8	+	+
Diazepam	202	238
Nordiazepam	218	481
THC	—	0.5
THC COOH	—	7.2

Table 2 Results of hair analysis of the subject 1 (ng/mg).

Compound	Hair strand (3 cm)
α-PVP	0.88
Oxo-PVP	< LOQ
Morphine	38
Cocaine	8
BZE	1.60
Norcocaine	0.50
Amphetamine	0.65
MDMA	0.05
Methadone	5
EDDP	0.45

Discussion

It should be noted the importance in quantity of products seized (more than 300 grams). One of the subjects admitted that this purchase on the darkweb was intended to achieve traffic in the form of capsules and they had already crafted a hundred.

Some of the symptoms presented by the one or the other subjects are comparable to those described in the literature [4,8—10]: clonies, dysarthria, tachycardia, euphoria, violent aggressive behavior... even if we cannot evaluate the part related to polydrug abuse. These effects are common to all synthetic cathinones.

The blood α-PVP concentrations measured for both subjects correspond to toxic levels as reported in the literature [4,9—14]. These high concentrations can be related to the consumption pattern of PVP: the subject 1 says having smoked α-PVP for 3 weeks and then be injected during the four days before his arrest. They are in accordance with determined concentrations of α-PVP in the literature. Adamowicz et al. [11] has reported in 2016 the ranges in specific type groups of cases as follows: DUID (driving under influence of drugs): 6.4—99 ng/mL; traffic accidents: 10.2—30 ng/mL; drug possession: < 1—98 ng/mL; non-fatal intoxication: 1.2—56 ng/mL; deaths: 1.1 ng/mL to 6200 ng/mL; and others: 2.6—136 ng/mL. The same author

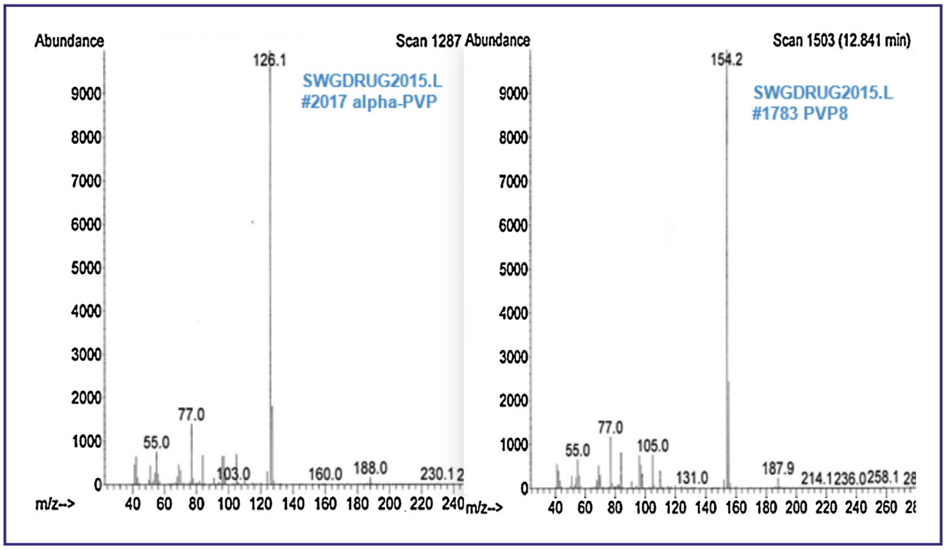

Figure 4. Mass spectra of α-PVP ($C_{15}H_{21}NO$; 231 g/mol) and PV8 ($C_{17}H_{25}NO$; 259 g/mol).

states that the effects of impairment were notable in a wide range of concentrations, but 40 ng/mL was proposed as the cutoff value for predicting significant influence of α-PVP on psychomotor performance. In 2016, Ferec et al. [10] describes 2 cases of non-lethal poisoning with hospitalization for agitation, hallucinations, tachycardia and hypertension. In these 2 cases, α-PVP and oxo-PVP (metabolite) have been identified in urine with concentrations of 11,880 (568) ng/mL and 15,840 (913) ng/mL, respectively. Eiden et al. and Frezil et al. [12,13] described 2 fatal intoxications with α-PVP concentrations of 1500 ng/mL (plasma) and 1020 ng/mL (peripheral blood). Three cases of α-PVP related deaths are reported by Richards-Waugh et al. [14] with concentrations of 100, 500 and 290 ng/mL where the drug was found to be the cause or a significant contributory cause to the death.

Analyzes performed in the hair of two subjects allows an evaluation of a major abuse history. The hair of the subject 1 (brown short hair) was collected 1 month after hospitalization. The hair of the subject 2 (blond long hair) was collected 4 months later, while the subject was in jail and hair length (20 cm) allowed doing segmental analysis.

In accordance with their confessions to police, analyzes for both subjects confirm the addiction to morphine. Both subjects admitted injecting regularly Skenan and taking methadone as part of a treatment for opiate substitution. Both have also admitted a high consumption of cocaine in the previous year. Consumption of other stimulants (amphetamine, MDMA) was more sporadic. Segmental hair analyses show that the subject 2 was exposed to α-PVP for at least 1 year. Analysis of the proximal segment (corresponding to the period in which the subject was in jail) shows the stop of drug abuse and regular intake of methadone.

Very few cases reported α-PVP concentrations in hair. Salamone [15,16] described sporadic findings in the literature: a concentration of 1.04 ng/mg in a 32 year old man known as polydrug abuser and concentrations from 7.4 to 9.4 ng/mg in segmental analysis in 4 subjects. Hair

Table 3 Results of hair analysis of the subject 2 (ng/mg).

Compound	Segment 1 (proximal 4 cm) Jail period	Segment 2 (4 cm) Events period	Segment 3 (4 cm)	Segment 4 (4 cm)
α-PVP	0.19	1.77	1.99	1.30
2-oxo-PVP	< LOQ	0.13	0.19	0.26
Morphine	19	70	75	85
Cocaine	3	11	11	12
BZE	2	4	5	7
Norcocaine	0.08	0.20	0.18	0.23
Amphetamine	0.65	1.68	1.74	2.26
Metamphetamine	< LOQ	0.08	0.11	0.15
MDMA	0.18	0.38	0.74	1.86
Methadone	8.99	2.84	1.93	1.68
EDDP	0.64	0.08	< LOQ	< LOQ

concentrations measured in the 2 subjects are in agreement with those values.

Conclusion

This report illustrates again the use of synthetic products easily accessible on the Internet and provides useful information regarding the toxicological data of cases involving designer stimulants, highlighting the need to consider the potential involvement of such drugs when presented with suggestive incident circumstances. The symptoms of these 2 cases were related to exposure to flakka (α-PVP), a cathinone derivate close to MDPV (methylenedioxypyrovalerone). We report here some clinical data, seized materials analysis, blood concentrations in a non-lethal poisoning but one of the main interest of this case is to document hair concentrations of α-PVP in a major drug abuse history.

Disclosure of interest

The authors declare that they have no competing interest.

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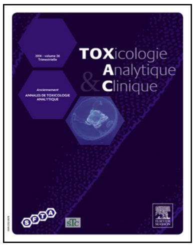

CASE REPORT

Identification of 25I-NBOMe in two intoxications cases with severe hallucinations

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Received 28 September 2016; received in revised form 25 November 2016; accepted 25 November 2016
Available online 29 December 2016

KEYWORDS
New psychoactive substances;
25I-NBOMe;
Plasma;
UPLC-MS/MS;
Severe hallucinations

Summary The abuse of new psychoactive substances (NPS) has been dramatically increasing all around the world since the late 2000s. The availability of hundreds of NPS in the past decade is challenging for both public health and global drug policies. We present the intoxication of two subjects who had sniffed a white creamy powder, characterized as 25I-NBOMe [2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine]. Plasma samples were analyzed by ultra-high performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS). Concentrations of 25I-NBOMe were 37 and 72 ng/mL, respectively. Major impairment of both subjects, including visual hallucinations and alteration of behaviour were attributed to these high concentrations. The data were compared to the few reports available in the medical literature.

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Introduction

For several years, new synthetic drugs appeared on the Internet. These are the new psychoactive substances (NPS), which designate a wide range of molecules having the same pharmacological effects as various illegal substances such as ecstasy, amphetamines, benzodiazepines, opiates, or cannabis. Their chemical structures can be close to the original

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http://dx.doi.org/10.1016/j.toxac.2016.11.004
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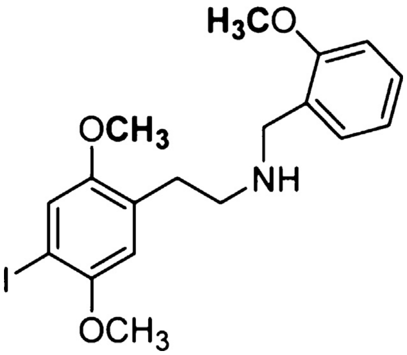

Figure 1. Chemical structure of 25I-NBOMe.

compound, but can be also very far from it. These substances can skirt the narcotic laws because they are not listed as controlled drugs. Generally sold on the internet, NPS are differentiated by their chemical names or trade names such as ''bath salts'', ''plant for nutrients'', ''incense''... According to a survey of the French Observatory Drugs and Drug Addiction [1], more than 382 substances circulated freely in the European Union between 2008 and 2015, including 176 in France. NPS are becoming more prevalent in the world of teenagers and parties, given the ease of purchase and use.

Among NPS, a group named phenethylamine NBOMe has gained importance in the recent years. N-Methyl-Benzyl-Oxy is the extension of the psychedelic family phenethylamine 2C, discovered by Dr. Alexander Shulgin and described in his book published in 1991 PIHKAL [2].

Among NBOMe, 25I-NBOMe (Fig. 1), or 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl) methyl]ethanamine, was discovered in 2003 by Ralf Heim [3] at the University of Berlin. It is a potent agonist of 5-HT2A receptors [4] in the brain. It can be consumed most often in the form of drops [5,6] or blotters and consumed by the transmucosal, sublingual or intranasal routes.

The NBOMe compounds have numerous psychedelic effects [5]: visual hallucinations, altered time and space, change of consciousness... They also have stimulating effects like euphoria or empathy. These effects can last from 5 to 10 hours [5]. They produce many side effects, including convulsions, paranoia, psychosis, coma up to death.

Analytically, the characterization of a consumption of 25I-NBOMe is not obvious. The literature is very poor in reporting cases where 25I-NBOMe was identified. Epidemiologically, only very few clinical cases of consumers were reported, indicating a low prevalence of this compound. Due to its possible misidentification, very few samples have been specifically tested for 25I-NBOMe. Indeed, consumers are generally accustomed to consume NPS and know how to manage their doses and the associated side effects [7]. Furthermore, for analysis of any NPS, it is necessary to have the pure standard substance to be able to build a reference spectrum and to identify the molecule in further biological specimens. Unfortunately, these standards are only available several months after the release of the substance on the Internet, and the large number of molecule is an economic problem to maintain an up to date reference library. The identification of 25I-NBOMe in blood is difficult, given that ingested doses are generally between 100 and 600 micrograms [8]. As a consequence, the use of tandem mass spectrometry appears as a pre-requisite to detect the very low circulating amount of the drug, in the sub ng/mL range and to entrance the specificity.

The objective of this publication is to present the analytical procedure established for assaying 25I-NBOMe in plasma of two subjects admitted at the Emergency room of the Hospital of Strasbourg.

Clinical case

Two gay men were discovered naked in their home. A friend of the couple called the medical rescue department because he found they acted strangely. Both reported visual hallucinations, incoherent speech, dysarthria and a significant aggressive behaviour. The emergency team noted a highly disinhibited behaviour and gestures of sexual nature (masturbation).

At their arrival at the hospital at 10:00 pm, both subjects reported having sniffed at 6:30 pm several drugs, ordered via the Internet. These drugs were methiopropamine, methylenedioxypyrovalerone (MDPV), amphetamines and opiates. Sildenafil was also abused by one of the addict, while the second reported alcohol consumption (two beers). A near death feeling was reported. Both individuals claimed to be regular consumers of NPS since 2014.

The clinical examination of the first patient highlighted a Glasgow score at 15, a coherent but highly productive speech, an absence of hallucinations, a reactive bilateral mydriasis, and a normal cardiopulmonary examination. The same clinical picture was observed for the second patient. The main symptoms were neurologic and psychiatric. Clinical observations went back to normal with appropriate behaviour around 3:00 am. After 5 hours of supervision, the two individuals were discharged from the hospital. A blood sample from each subject was collected and sent to the biochemistry laboratory for standard parameters (all within the normal range). Only 300 μL of plasma were available for the screening of 25I-NBOMe and MDPV at our laboratory.

During the first steps of rescue at home, the emergency team found three small plastic bags, each containing a white creamy powder. Two bags were labelled ''25I-NBOMe'' and the third was labelled ''MDPV''. A powder bag labelled 25I-NBOMe is presented on Fig. 2.

Materials and methods

The three bags of powder and a plasma sample from each patient were sent to the laboratory for 25I-NBOMe and MDPV analysis. There was no attempt to screen for other drugs, given the very low volume of specimens that were available (300 μL). A specific method to 25I-NBOMe was developed on an ultra-high performance liquid chromatography (Acquity class I) coupled to a Xevo TQD tandem mass spectrometer (UPLC-MS/MS) from Waters (Milford, MA, USA).

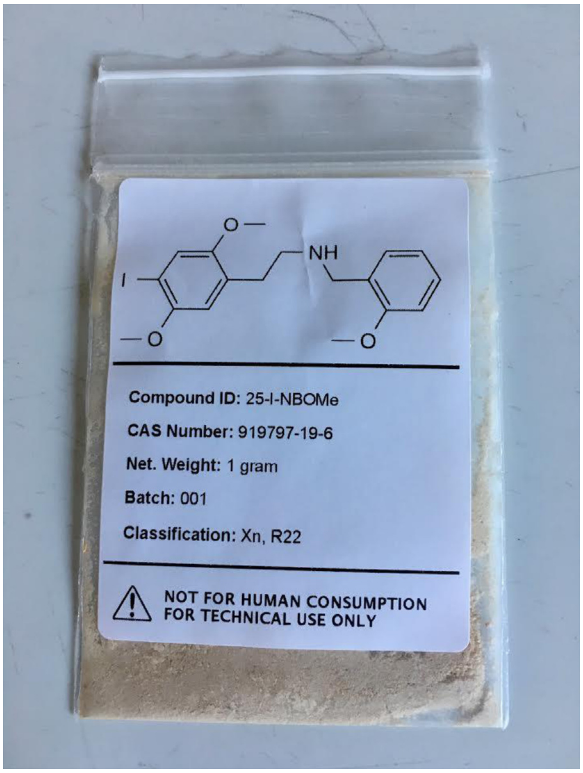

Figure 2. Picture of power bag annotated 25I-NBOMe.

Chemicals and reagents

Ammonium fomate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99-100%, AnalaR NORMAPUR and 36% hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Ethyl acetate for liquid chromatography and acetonitrile for LC-MS were purchased from Merck (Molsheim, France). MDMA-d_5 was purchased from LipoMed (Arlesheim, Switzerland) and diluted to appropriate concentration using acetonitrile.

25I-NBOMe analysis

Both powders labelled 25I-NBOMe were first tested by LC-MS over a mass range from *m/z* 50 to *m/z* 600 to check for purity. Only one peak was observed and the powder was then estimated to be pure. However, this statement was not verified by alternative methods, such as NMR. As a consequence, the concentration values should be considered as semi-quantitative, at best. An aliquot was prepared at 1 g/L in acetonitrile.

25I-NBOMe was extracted from 300 μL plasma in presence of 1 ng of MDMA-d_5 used as internal standard, with 300 μL borate buffer pH 9.5 and 2 mL of ethyl acetate. After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 30 μL of ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters HSS C18 column (150 × 2.1 mm × 1.8 μm) maintained at 50 °C in a thermostatically controlled enclosure. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile LC-MS (mobile phase B) as mobile phases at flow rate of 0.4 mL/min. The initial gradient was 80% mobile phase A and the final gradient, at 7 min, was 30% mobile phase A. An injection volume of 10 μL was used in all cases. A Xevo triple quadrupole mass spectrometer was used for the detection of the drugs. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of 25I-NBOMe and the internal standard: capillary voltage at 1.5 kV; source block temperature at 149 °C; desolvation gas nitrogen heated at 600 °C and delivered at a flow rate of 1000 L/h. In order to establish appropriate multiple reaction monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation of both species was performed. Collision energy was adjusted to optimize the signal for the 2 most abundant product ions of 25I-NBOMe: *m/z* 428.1 > 121.2 (CV: 22V) and 428.1 > 90.6 (CV: 36V), and the most abundant product ion of internal standard *m/z* 199.1 > 165.1 (CV: 12V). Transition *m/z* 428.1 to 121.2 was used for quantification of 25I-NBOMe. These transitions have already been described in the literature. MassLynx 4.1 software was used for quantification.

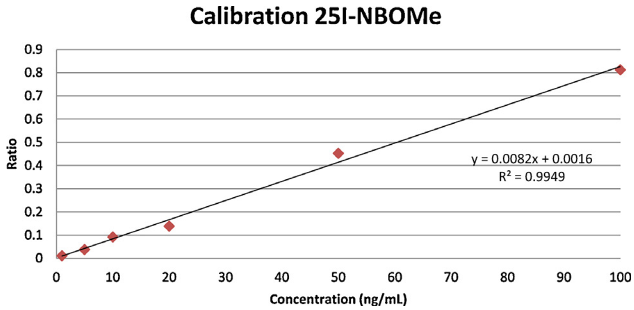

Figure 3. Calibration curve of 25I-NBOMe between 1 and 100 ng/mL.

Results and discussion

Three hundred microliters plasma was spiked with the powder found in the pocket of the patients. Linearity was observed for 25I-NBOMe concentrations ranging from 1 to 100 ng/mL, with a correlation coefficient of 0.9949. The calibration curve is presented on Fig. 3. The limit of detection was estimated to be 0.1 ng/mL, with a S/N ratio of 3. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in plasma (matrix effect < 20%, after analysis of 10 various blank plasma).

25I-NBOMe tested positive in the two plasma samples at 37 and 72 ng/mL. Chromatograms obtained after extraction of the plasma of both subjects are presented on Figs. 4 and 5. These concentrations are high in comparison with previous reports and may explain the initial effects of the drug observed in two individuals. MDPV was detected in the plasma of subject 1 at 59 ng/mL. The plasma of subject 2 was free of MDPV. No other drug was identified.

As indicated earlier, only few papers have reported the detection of 25I-NBOMe in human biological specimens. In 2014, Poklis et al. [8] reported a fatal case involving

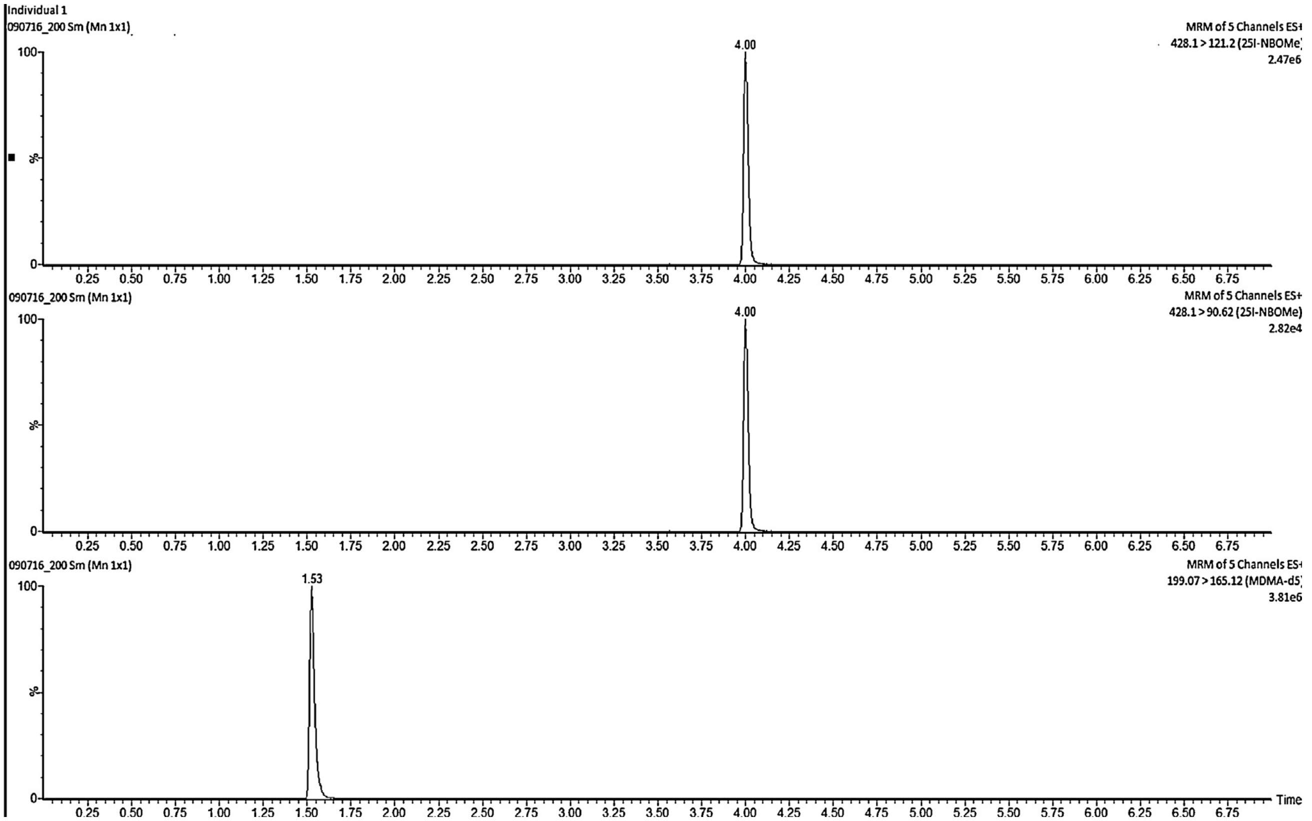

Figure 4. Chromatogram obtained after extraction of the plasma of subject 1. 25I-NBOMe concentration was 72 ng/mL. From top to bottom: 2 transitions for 25I-NBOMe (*m*/*z*: 428.1 > 121.2 and 428.1 > 90.62) and 1 transition for MDMA-d_5 (*m*/*z*: 199.07 > 165.12).

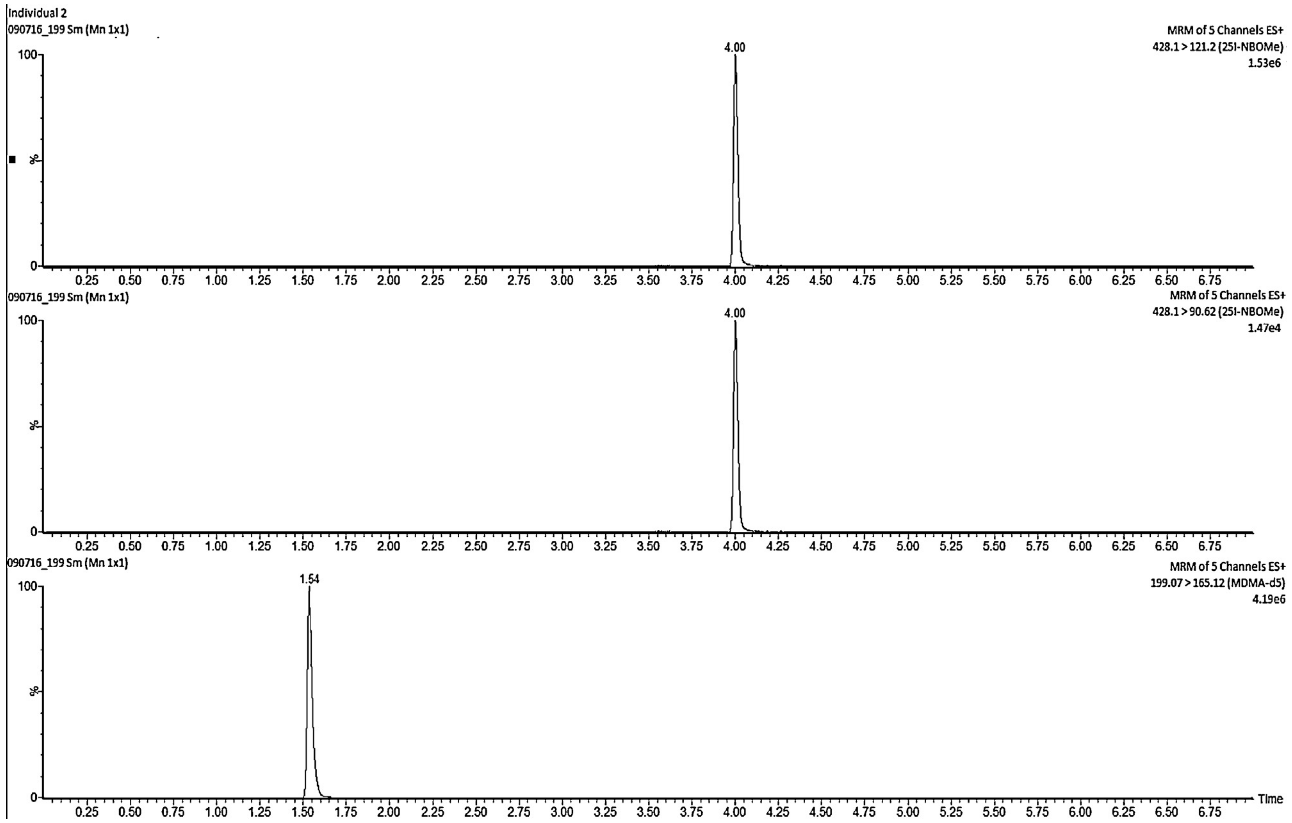

Figure 5. Chromatogram obtained after extraction of the plasma of subject 2. 25I-NBOMe concentration was 37 ng/mL. From top to bottom: 2 transitions for 25I-NBOMe (*m*/*z*: 428.1 > 121.2 and 428.1 > 90.62) and 1 transition for MDMA-d_5 (*m*/*z*: 199.07 > 165.12).

25I-NBOMe. A healthy 19-years-old mal ingested blotter paper infused with ''acid'' the day before his death by falling from his apartment balcony. Toxicological analysis of the decedent revealed 25I-NBOMe at 0.41 ng/mL in heart blood, 0.40 ng/mL in peripheral blood, 2.86 ng/mL in urine, 0.099 ng/mL in vitreous humor, 2.78 ng/mL in the brain, 5.64 ng/mL in the liver and 12.1 ng/mL in bile. It was established that hallucinations due to 25I-NBOMe have contributed to his fall.

In 2014, Boucher et al. [9] reported an intoxication case of a mixture of three NBOMe: 25I-NBOMe, 25C-NBOMe and 25H-NBOMe. A 29-year-old man, with schizophrenic and drug addiction histories, sniffed a drop of the mixture. After one hour, the subject developed signs of hallucinations, changes of behaviour, mydriasis, hypothermia and excessive sweating. Neurological failure caused coma and required invasive ventilation for 24 hours. Magnetic resonance imaging documented bilateral and symmetrical lesions of the basal ganglia. After one month, the patient had still memory impairment and significant abnormalities in executive functions. No blood was available for analytical investigations.

In 2015, Heiger et al. [10] described a series of self-reported exposures to 25I-NBOMe. Ten patients with an average age of 17 years (14—20 years), 8 males and 2 females, were admitted to a local emergency department after ingestion and/or insufflation of a drug referred to as ''25I''. Of 10 patients, six reported taking 25I alone; other substances were ethanol, 2,5-dimethoxy-4-ethylphenethylamine, marijuana and ketamine. Most common effects included tachycardia (90%), hypertension (70%), agitation (60%) and hallucinations (50%). Three patients required intubation, and all admitted patients were given intravenous benzodiazepines for sedation. The duration of toxicity was usually less than 24 hours in uncomplicated cases. Blood was analyzed in one single case, obtained from a patient presenting agitation and tachycardia. The serum 25I-NBOMe concentration was 0.76 ng/mL. All patients were discharge in good conditions once the symptoms were reversed.

Oosthuizen et al. [11] reported during the 2016 TIAFT meeting the case of the death of a 23-year-old female who was nasally insufflating an unknown white powder, which was believed to be synthetic LSD. Using LC-QToF, the following drugs were identified in her aortic blood: 28 ng/mL of 25I-NBOMe, 1 ng/mL of 25H-NBOMe and 0.7 ng/mL of 25C-NBOMe.

Shanks et al. [12] reported the deaths of two male teenage who consumed 25I-NBOMe and 25B-NBOMe in Indiana during 2014. 25I-NBOMe was detected in postmortem heart blood at 19.8 ng/mL for the second teenage.

Finally, Walterscheid et al. [13] have identified 25I-NBOMe in two fatalities without reporting the concentrations. In addition, in the same paper, they found in the serum of a 18-year-old mal presenting severe agitation and hallucinations a concentration of 0.76 ng/mL.

In all these cases involving a 25I-NBOMe intoxication [14], the same pharmacological effects were observed: alteration of behaviour, tachycardia, hypertension, agitation, aggression, visual and auditory hallucinations, mydriasis and excessive sweating. These symptoms have numerous forensic implications, as they can cause memory impairment, neurological and functional disorders.

25I-NBOMe is considered in France as a drug of abuse since a notice (*arrêté*) of 24th September 2015. More recently (27 September 2016), in the United States of America the Administrator of the Drug Enforcement Administration has placed 25I-NBOMe, including its optical, positional, and geometric isomers, salts and salts of isomers, whenever the existence of such salts, isomers, and salts of isomers is possible, into schedule I of the Controlled Substances Act.

Conclusion

We have presented 2 cases involving 25I-NBOMe poising with severe hallucinations and impairment. Symptoms spontaneously reversed after a few hours. Measured concentrations were high, in the light of the existing literature. Accordingly, the risk of missing 25I-NBOMe use or abuse during conventional toxicological screening is important. Since 25I-NBOMe appears not to be tested in many laboratories, one major concern about this drug regarding public health remains the lack of data on its potential acute toxicity and long-term side effects in humans. This is particularly true, as it has been reported in the recent literature [15,16] intoxication at a concentration lower than 1 ng/mL.

Disclosure of interest

The authors declare that they have no competing interest.

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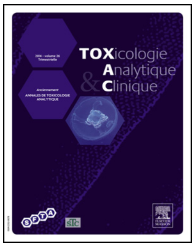

CASE REPORT

A fatal case of 3-methylmethcathinone (3-MMC) poisoning

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Received 15 November 2016; received in revised form 21 December 2016; accepted 22 December 2016
Available online 21 January 2017

KEYWORDS
3-methylmethcathinone;
Intoxication;
Isomer analysis

Summary Synthetic cathinones are one of the major pharmacological families of new psychoactive substances (NPS). We report a case of a young man who was found dead a couple of hours after sniffing substances bought on the Internet from Spain. 3-methylmethcathinone (3-MMC) was identified during general unknown screening by liquid chromatography with diode array detector and gas chromatography coupled to mass spectrometry. Quantification of 3-MMC was performed by gas chromatography tandem mass spectrometry after solid-phase extraction. Toxicological analysis of postmortem peripheral blood, cardiac blood, vitreous humor, bile and urine revealed 3-MMC in concentrations of 249 ng/mL, 609 ng/mL, 2988 ng/mL, 1291 ng/mL and 29,694 ng/mL, respectively. Powder and crystals seized at victim's home contained 32.9% and 36.9% of 3-MMC, respectively. No other xenobiotics were detected in postmortem samples. The cause of death was 3-MMC intoxication and the manner was accidental.

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http://dx.doi.org/10.1016/j.toxac.2016.12.010
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Introduction

Synthetic cathinones are related to the main psychoactive compounds of the khat plant. Since the 2000s, substances derived from cathinone have appeared on the European recreational drug market as alternatives to amphetamines [1]. The most widely encountered is 4-methylmethcathinone (4-MMC), also called mephedrone. To circumvent narcotic legislation, cathinone isomers and analogs were synthesized; more than 50 derivates were identified in Europe [2]. Two position isomers of mephedrone, 2-methylmethcathinone (2-MMC) and 3-methylmethcathinone (3-MMC), are available on the market. In France, mephedrone was classified as an illegal narcotic in 2010, and the generic cathinone family has been forbidden since July 2012 [3]. However, the French Observatory of Drugs and Drug Addiction (*Observatoire français des drogues et des toxicomanies* [OFDT]) reported 3-MMC and 2-MMC seizures in France, in 2014 and 2015, respectively [4]. Between 2012 and 2014, Cheze et al. [5] analyzed 64 cathinone seizures in France; two were identified as 3-MMC. Several fatal intoxications were reported in the literature involving 3-MMC associated to other drugs, such as 5-(2-aminopropyl)benzofuran (5-APB) [6] or gamma-hydroxybutyric acid (GHB) [7]. We here report a case of death exclusively involving 3-MMC, with quantification in the available postmortem matrices.

Case report

A 32-year-old man found dead at his home. According to some witnesses, after sniffing one or two lines of a white powder, the man suffered from headache and warm sensations. He lay down on a sofa at about 2:00 AM and was found dead at 7:00 AM. Some packets identified as 3-MMC containing crystals and a white powder, purchased on the Internet from Spain, were discovered in the victim's home. The deceased's personal history showed recreational GHB consumption. An autopsy was performed, and no sign of violence or any obvious cause of death was found. Full examination showed some non-specific indicators compatible with toxic death: major pulmonary edema associated with sero-hematic lung, and vascular congestion and edema, which spread to various organs. Several biological samples were collected for toxicological analysis: peripheral blood, cardiac blood, urine, vitreous humor (VH) and bile. Blood samples were collected in tubes with preservative (sodium fluoride). Samples were stored at 4 °C prior to analysis.

Material and methods

Standards and reagents

Certified stock solutions of mephedrone (4-MMC) at a concentration of 1 mg/mL, mephedrone-d3 and hydroxybutyric acid-d6 (GHB-d6) at 0.1 mg/mL were purchased from Euromedex (Souffelweyersheim, France). 2-MMC and 3-MMC at a concentration of 1 mg/mL were provided by LGC Standard (Molsheim, France). Methanol (MeOH), hydrochloric acid 1 M (HCl), ethyl acetate (EA), isopropanol (IPA), acetonitrile (ACN), sulfuric acid 95—97%, and ammonia 25% (NH_4OH) at HPLC grade were obtained from V.W.R (Fontenay-sous-Bois, France). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) 1% chlorotrimethylsilane (TMCS) was purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Drug-Screen® immunochromatographic assay for amphetamine, methamphetamine, opioids, cannabinoids, cocaine, 3,4-methylenedioxymethamphetamine (MDMA), and buprenorphine were purchased from Nal Von Minden (Regensburg, Germany). Strata X Drug B solid-phase extraction (SPE) cartridges (3 mL, 60 mg) were purchased from Phenomenex (Le Pecq, France). Water was dispensed by a PurelabElga® purifier (Veolia Water, Solutions & Technologies, Décines, France). Mephedrone-d3 was used as internal standard (IS) at a concentration of 10 ng/μL in methanol. For calibration standards and quality control (QC), appropriate dilutions were made from the solutions of 4-MMC, 3-MMC and 2-MMC at 1 mg/mL.

Routine toxicological analysis

Immunological screening was performed on urine. Quantitative analysis for ethanol was performed on peripheral blood and urine, using a headspace (Triplus) gas chromatograph (Trace GC) coupled to a mass spectrometer (DSQ II) (Thermo Electron, Courtaboeuf, France). General unknown screening (GUS) was performed after liquid-liquid extraction on urine by gas chromatography (7890B, Agilent Technologies, Massy, France) coupled to mass spectrometry (5977A, Agilent Technologies) (GC-MS), and on cardiac blood by liquid chromatography with a diode array detector (LC-DAD) (1100 Series, Agilent Technologies). LC-DAD conditions were previously described [8]. For GC-MS assay, the injector in splitless mode was set at a temperature of 280 °C. Separation was performed on an HP-5MS capillary column (length 12 m, 0.18 mm ID, film thickness 0.18 μm; Agilent). The initial oven temperature of 90 °C was held for 0.85 min, then increased by 21.5 °C/min to 200 °C, followed by a rate of 16 °C/min to 300 °C, held for 11 min; total run time was 23.2 min. Helium was used as carrier gas at a constant flow of 0.7 mL/min. The transfer line was set at 300 °C. Molecules were identified using homemade and commercial libraries: SWGDrug 2015, Pfleger 2011, and Wiley 2007 for GC-MS using macro-command [9].

Quantitative analysis of GHB

Blood, urine, and bile samples (200 μL) were spiked with 15 μL GHB-d6 at 100 ng/μL as IS. A total of 100 μL sulfuric acid (0.05 M) was added, followed by 3 mL EA. After 10 min agitation, the mixture was centrifuged for 10 min at 2000 g. The supernatant was evaporated to dryness under nitrogen stream at room temperature. Dry residues were derivatized by adding 30 μL BSTFA 1%TMCS (30 min at 80 °C). One microliter of sample was injected in splitless mode in a GC-MS using a 6890 N GC system coupled to a 5975 MS detector (Agilent Technologies). The injector was set at 250 °C. Separation was performed on an HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent). The initial oven temperature of 60 °C was held for 2 min, then increased by 20 °C/min to 260 °C; total run time was 12 min. Helium was used as carrier gas at a constant flow of 1.1 mL/min. The single-ion monitoring (SIM) mode was used with 1 quantifier ion (in bold) and 2 qualifiers to identify and quantify GHB (117, **159**, 233) and GHB-d6 (120, **165**, 239).

Quantitative analysis of 3-MMC

A specific method was developed then validated in all the four matrices, to separate and quantify the 3 position isomers: 4-MMC, 3-MMC and 2-MMC. Blood, urine, VH and bile samples (1 mL) were spiked with 20 μL IS solution. 2 mL sodium acetate buffer (100 mM, pH = 5) was added, followed by 2 mL MeOH/ACN (85:15 v/v). After 10 min agitation, the mixture was centrifuged for 10 min at 2000 g. The supernatant (approximately 4 mL) was purified on an SPE cartridge. Two washing steps were performed (1 mL HCl 0.1 M, then 1 mL ACN/HCl 0.1 M (70:30 v/v)) prior to elution by 2 mL EA/IPA/NH_4OH (70:20:10 v/v/v). Two hundred microliters MeOH/acetic acid (98:2 v/v) solution was added to the extract before evaporation to dryness under nitrogen stream at 50 °C. Dry residues were acetylated by adding 60 μL acetic anhydride and 40 μL pyridine (30 min, at 60 °C), evaporated again to dryness and reconstituted in 40 μL EA. One microliter of sample was injected in splitless mode in a gas chromatograph coupled to a tandem mass spectrometer (GC-MS/MS) using a 7890A GC system coupled to a 7000 Triple Quadrupole GC/MS detector (Agilent Technologies). The injector was set at 260 °C. Separation was performed on an HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent). The initial oven temperature of 60 °C was held for 1 min, then increased by 15 °C/min to 140 °C, followed by a rate of 30 °C/min to 212 °C; this temperature was held for 3 min then increased by 70 °C/min to 300 °C; total run time was 20 min. Helium was used as carrier gas at a constant flow of 1 mL/min. The transfer line was set at 320 °C. Three transitions in multiple reaction monitoring (MRM) mode, 1 quantifier (in bold) and 2 qualifiers, were used to identify and quantify 4-MMC and its isomers (**219 → 100**; 219 → 148; 119 → 91) and mephedrone-d3 (103 → 61; **222 → 103**). Retention time for 2-MMC, 3-MMC, mephedrone-d3, and 4-MMC was 10.41 min, 10.49 min, 10.73 min and 10.75 min respectively. All samples were quantified using matrix-matched calibration. Samples exceeding the upper limit of quantification were reanalyzed after dilution in phosphate buffer.

Validation procedure

Following Peters et al. [10], for methods used for publication of case reports, validation of the assay was performed on blood, focusing on linearity, selectivity, accuracy and precision. Calibration curves (50.0, 250, 500, and 1000 ng/mL) were traced by linear least-squares regression, using the MassHunter Workstation software for quantitative analysis, based on peak area ratio (analyte/IS) as a function of analyte concentration. Five blood samples of different origins were analyzed to assess method selectivity. Precision and accuracy were determined by analysis of 5 replicates at 3 concentrations: lower limit of quantification (LLOQ) at 50.0 ng/mL, low QC at 100 ng/mL, and high QC at 700 ng/mL. Acceptance criteria were set according to FDA guidance for validation of bioanalytical methods [11]: precision within 15%, except at the LLOQ (20%) and accuracy between 85% and 115% (80—120% at the LLOQ).

Table 1 3-methylmethcathinone concentration (ng/mL) in different matrices.

Matrices	Concentration (ng/mL)
Peripheral blood	249
Cardiac blood	609
Vitreous humor	2988
Bile	1291
Urine	29,694

Powder and crystal analysis

Five milligrams of powder or crystals was dissolved in 5 mL MeOH. After 10 min agitation, the mixture was centrifuged at 2000 g to eliminate insoluble components. One microliter and 50 μL supernatant were injected respectively in the GC-MS and LC-DAD according to GUS conditions and 1 μL in the GC-MS to determine GHB. For 3-MMC quantitative assay, 40 μL supernatant was added to 20 μL mephedrone-d3 solution at 10 ng/μL. Evaporation, acetylation and analysis by GC-MS/MS were performed as described previously.

Results

Toxicological analysis

Immunological screening on urine was negative for all narcotics tested. Ethanol was negative on peripheral blood and on urine. GUS on cardiac blood by LC-DAD and on urine by GC-MS highlighted the presence of 3-MMC. No other xenobiotic was detected. 3-MMC was quantified in all the matrices available from autopsy by specific GC-MS/MS assay. 3-MMC concentrations are presented in Table 1. Determination of GHB enabled quantification in peripheral blood, cardiac blood, bile and urine, at 35.7 μg/mL, 25.0 μg/mL, 12.4 μg/mL and 8.83 μg/mL, respectively. Screening on crystals and powder showed only presence of 3-MMC; no other narcotics or GHB were identified in the two substances. The crystal and powder composition in 3-MMC was respectively 36.9% and 32.9%.

Validation results

A linear model with a 1/x weighting factor was applied to 2-MMC, 3-MMC and 4-MMC calibration. Linear regressions were characterized by a determination coefficient (r^2) > 0.99. Selectivity was assessed as none of the five blank samples showing interference peaks at the retention time of the three compounds. Low QC and high QC precision and accuracy met the defined criteria, and are reported in Table 2. LLOQ was validated at 50.0 ng/mL, as precision and accuracy were satisfactory (Table 2).

Table 2 Assessment of accuracy and precision of 2-MMC, 3-MMC and 4-MMC at LLOQ, low QC and high QC on blood. Data from five replicates for each concentration.

	Spiked concentration (ng/mL)	Found (mean ± SD)	Precision (%)	Accuracy (%)
2-MMC				
LLOQ (*n* = 5)	50.0	55.2 ± 7.5	13.6	110
Low QC (*n* = 5)	100	88.3 ± 4.7	5.27	88.4
High QC (*n* = 5)	700	601 ± 68	11.4	85.8
3-MMC				
LLOQ (*n* = 5)	50.0	50.2 ± 3.8	7.56	100
Low QC (*n* = 5)	100	95.0 ± 2.4	2.51	94.9
High QC (*n* = 5)	700	739 ± 47.3	6.41	106
4-MMC				
LLOQ (*n* = 5)	50.0	49.2 ± 3.4	7.00	98.4
Low QC (*n* = 5)	100	108 ± 2.7	2.48	108
High QC (*n* = 5)	700	716 ± 19.8	2.76	102

LLOQ: lower limit of quantification; QC: quality control; SD: standard deviation.

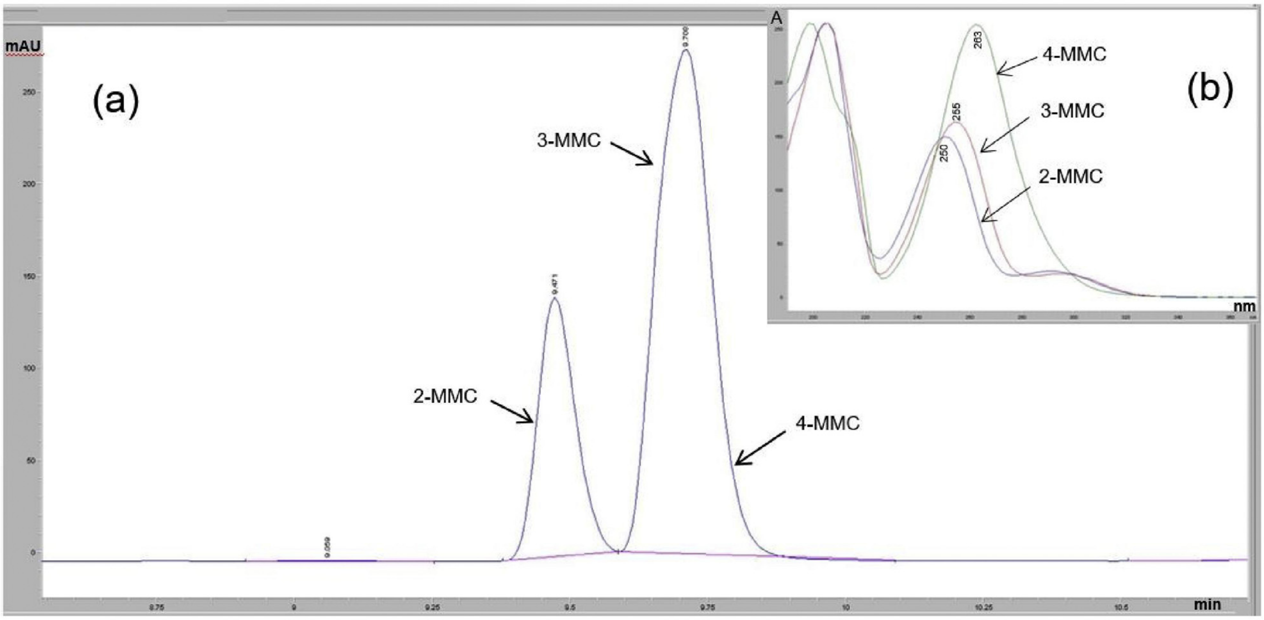

Figure 1. a: chromatogram of isomers (2-MMC, 3-MMC, 4-MMC) at 210 nm under LC GUS conditions; b: UV spectrum of each isomer with maximum absorbance in nm.

Discussion

We report a case of fatal poisoning in a young man who sniffed 3-MMC powder (purity 32.9%) bought on the Internet. Few cases of intoxication involving 3-MMC have been reported in the literature. But as underlined by Bäckberg and coworkers [12], the difficulty of distinguishing 3-MMC from 4-MMC may lead to the real number of cases of 3-MMC use and its toxicity being underestimated. The 3 isomers of methylmethcathinone (MMC) can be differentiated by their UV spectra in LC-DAD; they had the same profile of absorbance but different maximum absorbance: 250 nm for 2-MMC, 255 nm for 3-MMC and 263 nm for 4-MMC, as illustrated in Fig. 1. The drawback of LC-DAD is that, if method resolution is insufficient, the isomers may coeluate. This was the case with our liquid chromatography GUS conditions (Fig. 1). On the contrary, in our gas chromatography method, the three compounds were fully separated, as illustrated in Fig. 2, with retention times of 5.35 min for 2-MMC, 5.40 min for 3-MMC and 5.55 min for 4-MMC. The slight time difference highlighted the importance of being attentive, as mass spectrum is not discriminating: for these 3 structural isomers, which differ only in methyl position on the cyclic group, mass spectrum on GC-MS and MRM transitions on tandem mass spectrometry (MS/MS) were identical. Obviously, position isomers will have the same mass on high-resolution mass spectrometry, and chromatographic separation is therefore required. Tests were performed using ion mobility

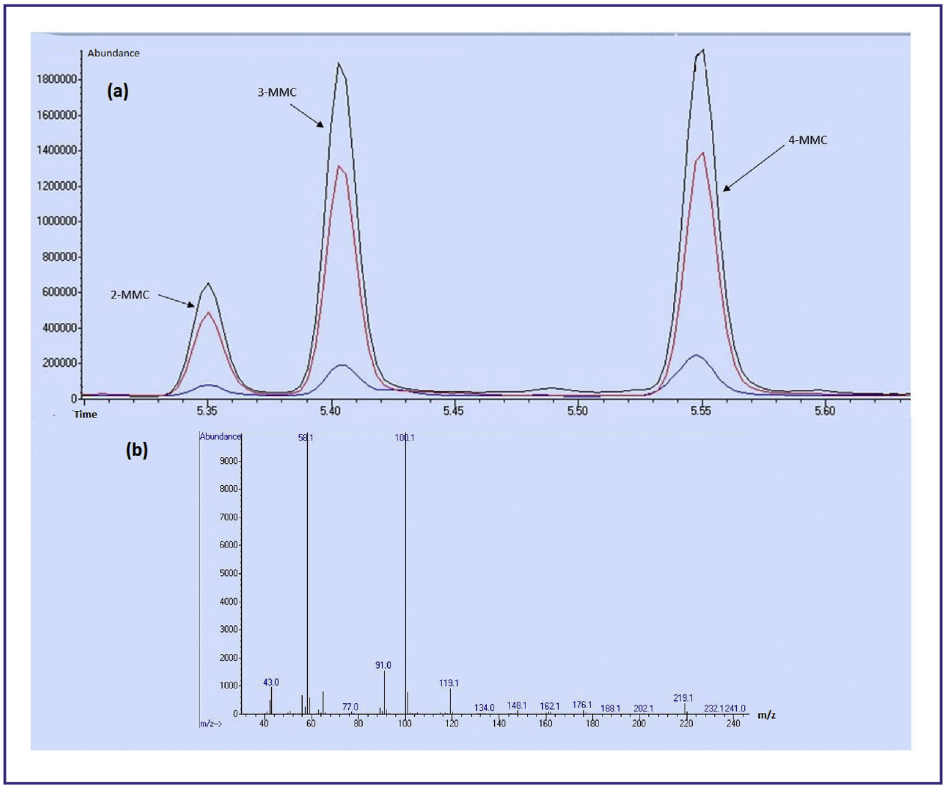

Figure 2. a: extract ion chromatogram of isomers (2-MMC, 3-MMC, 4-MMC) in gas chromatography under GUS conditions; b: mass spectrum common to the three isomers. Legend of extract ion chromatogram: m/z 58 in black; m/z 91 in blue; m/z 100 in red.

spectrometry, an advanced technology able to separate and identify ionized molecules based on their mobility in a carrier buffer gas. Results were not satisfactory for discrimination of 2-MMC, 3-MMC and 4-MMC (data not shown). One hypothesis is that the structures are too spatially close to be differentiated from one another. In conclusion, LC-DAD and GC-MS enable MMC isomers to be distinguished, thanks to UV spectra and retention times respectively.

In the present case, among the available postmortem matrices, 3-MMC concentrations were lowest in blood, with 249 ng/mL for peripheral blood and 609 ng/mL for cardiac blood. Other cases of fatal intoxication involving 3-MMC were found in the literature. Jamey et al. [7] reported a fatal case with blood concentrations of 330 ng/mL and 910 ng/mL in peripheral and cardiac blood respectively, associated with lethal concentrations of GHB. In Adamowicz et al.'s study of 95 cases of 3-MMC consumption [13], blood concentrations in 5 fatal and 1 non-fatal case ranged from < 1 ng/mL to 1600 ng/mL (mean 276.2 ng/mL, median 16 ng/mL); in these cases, death was not attributable solely to 3-MMC. This study also reported 66 cases of driving under the influence of the drug (3-MMC blood concentration range: 1 to 171 ng/mL), 4 traffic accidents (< 1 ng/mL to 29 ng/mL), 9 drug possessions (2 ng/mL to 408 ng/mL) and 10 other offenses (< 1 ng/mL to 61 ng/mL). Férec et al. [14] reported three cases of non-fatal intoxication, with 3-MMC plasma concentrations of 7 ng/mL, 220 ng/mL and 304 ng/mL. The highest concentrations in these non-lethal cases were quite similar to those in fatal cases. This large range of concentrations from lethal and non-lethal cases of 3-MMC consumption reveals the difficulty of determining a toxic concentration threshold. Various factors may explain this. Human pharmacodynamic and pharmacokinetic data are sparse for synthetic cathinones and non-existent for their isomers. Forum data describing short-term 3-MMC effects [13] and 3-MMC pharmacokinetic profile in pigs (plasma half-life 0.8 h after oral administration [15]) suggest quick blood elimination of 3-MMC. Thus, the interval between intake and sampling or death is decisive for interpretation. Moreover, 3-MMC was generally consumed in association with other drugs of abuse. For example, in 80% of cases (*n* = 76 out of 95), 3-MMC was associated with other substances such as amphetamine, tetrahydrocannabinol (THC) or various new psychoactive substances (NPS) [13]. These associations can interfere with the interpretation of specific 3-MMC effects, toxicity and concentration. Since few fatal cases related to this substance were published, concentration interpretation is limited. Adamowicz et al. studied reported drug consumption symptoms in a population of drivers under the exclusive influence of 3-MMC: blood concentrations in drivers without symptoms (3 to 171 ng/mL) were higher than in the group presenting drug effects such as agitation, verbosity, uncoordinated movement, etc. (11 to 19 ng/mL) [16]. According to the authors, high concentrations with few symptoms could be the result of chronic consumption and tolerance. This tolerance phenomenon has been described for 3,4-methylenedioxymethamphetamine (MDMA) [17]. Another problem in 3-MMC concentration interpretation is the instability of the molecule in biological samples, which may lead to underestimation of blood concentration. In the present case, due to the delay in obtaining customs permission for importation of 2-MMC and 3-MMC, quantitative analyses were undertaken only after 2 months; during this period, postmortem samples were stored at 4 °C. Jamey et al. [7] observed that 3-MMC blood concentration decreased by more than 70% in 14 days at 4 °C. Thus, 3-MMC degradation during the 2-month interval between collection and quantitative analysis cannot be excluded, and indeed seems likely in view of the vitreous humor concentration (2988 ng/mL), which was 12-fold higher than in peripheral blood. This high VH-to-blood ratio may be explained by greater stability of 3-MMC in VH than in blood, as reported for cocaine and 6 monoacetyl-morphine [18]. To our knowledge, this is the first 3-MMC concentration to be reported in VH, and no data on stability and pharmacokinetics in this matrix are available. However, other hypotheses could also explain this high vitreous concentration: e.g., 3-MMC vitreous pharmacokinetics, contamination when sampling, such as choroid and retinal cell aspiration, etc. [19].

Urine was the sample with the highest 3-MMC concentration, at 29,694 ng/mL. Some cathinones give immunological screening positive response for amphetamine, or methamphetamine, or MDMA, such as 4-methylethcathinone, which gives a cross-reaction with these 3 types of immunoassay [20]. In this case, immunological screening was negative, despite the high concentration of 3-MMC in urine. When urine is unavailable in fatal intoxication cases, Marinetti et al. [21] suggested using bile as a good matrix to screen for synthetic cathinones. In the present study, the bile concentration (1291 ng/mL) was much lower than in urine. Jamey and coworkers observed the same phenomenon, with bile and urine 3-MMC concentrations of 580 ng/mL and 29.02 μg/mL, respectively. These results suggest lower bile excretion than urinary elimination, as previously reported for other cathinones such as mephedrone, where blood, urine and bile concentrations were respectively 1.33 mg/L, 144 mg/L and 1,29 mg/L [22], or methylenedioxypyrovalerone (MDPV), where blood, urine and bile concentration were respectively 0.44 μg/mL, > 5 μg/mL and 0.88 μg/mL [23]. Since recreational use of GHB was hypothesized by the police, GHB was investigated in the different matrices collected during autopsy, except in VH, for which the available volume did not allow measurement of GHB. In the studies by Kintz et al. [24] and more recently by Andresen-Streichert et al. [25], GHB concentrations in the different samples (35.7 μg/mL, 25.0 μg/mL, 12.4 μg/mL and 8.83 μg/mL in peripheral blood, cardiac blood, bile and urine, respectively) did not allow any conclusion as to an exogenous source of GHB. As reported by Delannoy et al. [26], death related to synthetic cathinones is linked to co-consumption or to cardiac disorders, implicating the catecholaminergic reaction generated by the cathinones. This reaction is particularly important in case of pre-existing cardiac disorder. In the present case, there was no co-consumption and no cardiopathy was observed, neither on macroscopic or anatomopathological examination. Moreover, reported as one of the symptoms intoxication of 3-MMC by Marinetti et al. [21], the warm sensation whose the victim complained to witness before lying down and deceased, reinforced this hypothesis. Indeed, cathinones are derivatives of phenethylamine, like which they act as central nervous system stimulants. They decrease serotonin transporter function, causing excessive serotonin activity, which can result in hyperthermia, as in neuroleptic syndrome [27,28].

Conclusion

According to the circumstances of death, autopsy observations and toxicological results, the present cause of death was toxic, exclusively involving 3-MMC, and the manner was accidental.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgements

The authors appreciate to thank Waters France to their help to test the ion mobility method to analyze the tree isomers studied.

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CASE REPORT

Presence of mephedrone and methylone in an attempted suicide: A surprising result where toxicological analyses have changed the initial conclusions

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Reçu le 10 novembre 2016 ; reçu sous la forme révisée le 4 janvier 2017 ; accepté le 4 janvier 2017
Disponible sur Internet le 15 février 2017

KEYWORDS
Methylone;
Mephedrone;
Cathinones;
Forensic toxicology

Summary Mr. D., aged 31, was found lying unconscious on his back on the grounds of his residence with bed sheets wrapped around his neck and attached to a tree branch for the purposes of suicide by hanging. Emergency services arrived at his residence where a Glasgow Coma Scale score of 6 led to tracheal intubation. The cardiovascular examination showed tachycardia. Blood tests performed on admission revealed hyperglycemia (3.80 g/L), metabolic acidosis, alcohol concentration at 0.16 g/L, CRP at 110 mg/L and transaminases (AST) at 84 IU/L. A bag of white powder and an empty vodka bottle were found at the patient's residence, suggesting simultaneous use of alcohol and cocaine. Therefore, a charge of illegal drug possession was filed by the police and a blood specimen was collected to test for alcohol, drugs of abuse and medications. The forensic tests on Mr. D.'s blood, performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), revealed the presence midazolam (not quantified), methylone (936 ng/mL), and mephedrone (116 ng/mL). Other tests were negative, including cocaine. Concomitant consumption of alcohol and cathinone may induce coma, associated to hyperglycemia. This case, with detection of illegal drugs by an expert laboratory using standard techniques is shown here to be critical to understanding the clinical effects of suspected drugs; this type of analysis must be prescribed rapidly once the patient is hospitalized (short half-lives and stability problems of mephedrone).

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http://dx.doi.org/10.1016/j.toxac.2017.01.004
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Introduction

Since 2000, all new synthetic recreational drugs have been referred to as new psychoactive substances or NPS, which include cathinones. The cathinone category includes the synthetic derivatives of a natural substance known as S-cathinone. Cathinone is found in khat, or *qat* (*Catha endulis*), a plant native to the Horn of Africa.

Substitution of one or more functional groups results in the synthesis of cathinones, such as methylone and mephedrone. Structurally similar to amphetamines, they are phenylethylamines, with a ketone in the beta position [1].

New designer drugs are synthesized by clandestine laboratories (mainly in continental Asia) due to an increasing request of the black market. Consumers may order these substances via the Internet since there are no specific administrative formalities with regard to exportation.

Legislation in European countries concerning classification of these substances as illegal drugs to prohibit use is highly variable from country to country. In France, methylone and mephedrone are classified as illegal drugs since 2010.

The effects of these two cathinones are poorly described in the literature and are difficult to distinguish from the effects of amphetamines or cocaine. Pharmacological activity is apparent after 15 minutes and may last up to 3 hours [2,3]. Users may prolong effects by taking multiple doses over several hours [4].

The mechanism of action is the same for all S-cathinone derivatives: inhibition of monoamine transporters for dopamine, serotonin and adrenaline in the central nervous system. In addition, there is increased presynaptic release of catecholamines such as noradrenaline [3,5]. In a recent review [6], Karila et al. have discussed the effects and risks associated to mephedrone and methylone and have pointed important health-related issues in relation to the somatic, psychiatric and addictive consequences of their use.

Mephedrone intoxication, already described in the literature [7], is characterized by sympathomimetic toxicity: agitation, mydriasis, tachycardia, hypertension and more rarely arrhythmia and convulsions. Patients treated by emergency services/departments following mephedrone use often present the following symptoms: agitation, sweating, headache, palpitations and nausea [8]. Regan et al. [9] reported 89 emergency admissions following mephedrone consumption, for the period from 2009 to April 2010, with the following symptoms: agitation, chest pain, paresthesia, palpitations and shortness of breath.

Psychological complications and the risk factors for dependence are still poorly understood. The comedown syndrome after mephedrone consumption is similar to withdrawal, involving shaking, shivering, lowered body temperature and paranoia. Very little information has been published on methylone.

In this report, we present a case of combined mephedrone and methylone intoxication related to a suicide attempt.

Case description

This laboratory was requested by a police officer to perform a blood screening for alcohol, pharmaceuticals and drugs of abuse collected from a subject who wanted to commit suicide while under the influence of psychoactive substance(s). Although the laboratory did not receive the medical history of the subject, some informations were available, including the initial report of his hospitalization.

Mr. D., aged 31, was found lying unconscious on his back on the grounds of his residence with bed sheets wrapped around his neck and attached to a tree branch for the purposes of suicide by hanging. Emergency services were called and they arrived at his residence at 10:20 am. The subject was then hospitalized in intensive care unit. Due to a Glasgow Coma Scale score of 6, a tracheal intubation was achieved. Cardiovascular examination revealed tachycardia.

Blood tests performed on admission by the laboratory of the hospital revealed a hyperglycemia (3.80 g/L), metabolic acidosis, an alcohol concentration at 0.16 g/L, CRP at 110 mg/L and AST at 84 IU/L. Rhabdomyolysis (CPK = 2278 IU/L) was observed during hospitalization.

During the early hours of hospitalization, police went at his home where a bag of white powder and an empty vodka bottle were found. As a consequence, a charge of illegal drug possession was filed. It was the opinion of the police that the subject has simultaneously used alcohol and cocaine. The Prosecutor of the city ordered blood collection for forensic purposes. This specimen was obtained the same day around 4:00 pm. This is the specimen that was analyzed by the laboratory.

The patient's medical history revealed that he had consumed alcohol at around 4:00 am to have the courage to hang himself. The patient's discharge documents refer to a suicide attempt involving cocaine and alcohol intoxication and a failed hanging (absence of marks).

The patient's neurological status improved, with recovery of normal waking consciousness a few hours after admission. He was calm and cooperative. Psychiatric treatment was recommended. Three days later, the patient succeeded in hanging himself. However, the laboratory was not informed about possible new toxicological analyses. In particular, it was not possible to obtain a hair specimen, which would have been suitable to establish whether consumption of cathinones was repetitive.

Materials and methods

Tests to detect pharmaceuticals and illegal drugs (excluding cannabis) were performed with an ultra-high performance liquid chromatography system coupled with a quadrupole mass spectrometer (UPLC-Quattro micro, Waters). The chromatographic column was an Acquity HSS C18 1.8 μm (2.1 × 150 mm). The mobile phase consisted of an ammonium formate buffer (5 mM, pH 3.0) and an acetonitrile solution acidified with 1 % formic acid.

Methylone and mephedrone were extracted from 1 mL of whole blood with MDEA-d_5 used as the internal standard, under alkaline conditions, with a dichloromethane/isopropanol/n-heptane mixture (30:15:55, v/v/v) and borate buffer adjusted to pH 9.5.

After evaporation of the organic phase, the dry extract was dissolved in 100 μL of mobile phase, and 20 μL were injected into the chromatographic system.

Cannabis (THC and metabolites) was tested by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS).

Common pharmaceuticals were extracted from 1 mL of whole blood with diazepam-d_5 used as internal standard, under alkaline conditions, by a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50/30/20/0.5).

The characteristic ions for the substances are as follows: methylone (m/z 208.3 > 160.2, 208.3 > 132.1) and mephedrone (178.3 > 145.2, 178.3 > 160.2).

Performance characteristics for the method are as follows:

- For mephedrone: linearity: 10 to 2000 ng/mL with precision/CV of 12 % (low values) and 13 % (high values) and R2 > 0.98, LOD of 2 ng/mL and limit of quantification (LOQ) of 10 ng/mL;
- For methylone: linearity: 10 to 2000 ng/mL with precision/CV of 3 % (low values) and 13 % (high values) and R2 > 0.98, LOD of 2 ng/mL and limit of detection (LOQ) of 10 ng/mL.

Results and discussion

The concentration measured in blood was 936 ng/mL for methylone and 116 ng/mL for mephedrone. These concentrations are high relative to concentrations generally observed in emergency departments [6,10].

Midazolam was also observed during the screening process but not quantified. This was the consequence of administration during the early hours of hospitalization.

No other drug was identified. Cocaine, benzoylecgonine, cocaethylene and norcocaine were not detected. The laboratory did not receive the bag containing the white powder and it is unknown if it was tested.

Alcohol consumption was assumed to have occurred the previous night, at around 4 am. It is therefore not surprising to observe a negative result in the specimen collected at 4 pm the same day.

It is highly likely that the mephedrone concentration was underestimated. Even with storage at 4 °C, the molecule's instability in vitro has been described [11]. A new measurement of these two molecules was performed 16 months later (storage at 4 °C) under the same analytical conditions: mephedrone was no longer detectable and methylone had fallen to 48 ng/mL. Analyses should therefore be performed as soon as possible after samples are collected and analytical toxicologists are encouraged to store the specimens at −20 °C.

Given the lack of further requests (the laboratory did not receive the postmortem specimens and it is even not know if an autopsy was performed), it is difficult to establish the relation with cathinones consumption and the suicide attempt. Various hypothesis can occur: bad trip, massive impairment preventing adequate hanging, difficult down period... The lack of hair collection is also very disappointing, as this matrix would have helped in establishing the pattern of drug use of the subject.

Obviously, this case demonstrates the importance in testing all forensic specimens for NPS. The initial feeling of the police was cocaine abuse but the laboratory demonstrated that other psychoactive substances were involved. Immunological tests would have failed in identifying both methylone and mephedrone, clearly demonstrating the power of LC-MS/MS.

Conclusion

In the case described, two cathinones were found at blood concentrations of 936 ng/mL for methylone and 116 ng/mL for mephedrone. These concentrations may be underestimated due to the poor in vitro stability of these two new synthetic products. Ingestion of the two molecules with alcohol consumption resulted in coma (Glasgow Coma Scale score of 6) with adrenergic storm. The subject's comatose state played a role in the failure of his attempted suicide by hanging.

Analysis of blood samples by an expert laboratory with standard techniques to detect illegal drugs and medications was of prime importance in understanding the clinical effects of the suspected substances; such analysis must be prescribed rapidly after hospitalization (short half-lives and instability problems in the case of cathinones).

Disclosure of interest

The authors declare that they have no competing interest.

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CASE REPORT

''Synthacaines'': A mosaic of substances for a wide range of effects, from a case

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Received 15 November 2016; received in revised form 9 January 2017; accepted 9 January 2017
Available online 15 février 2017

KEYWORDS
Synthacaine;
Methiopropamine;
2-amino-indane;
Paranoia;
Dysmorphophobia;
N-methyl-2-amino-indane

Summary In recent years, a class of new designer drugs commercialized under the generic name of ''Synthacaines'' have been available both to the surfaceweb and the illicit drug market or deep web. We report the case of a 30-year-old man admitted to the psychiatry department for acute anxiety crisis with paranoid experiences associated with dysmorphophobia, who followed one week of intensive use of ''synthacaine'' he bought on the web. Toxicological analysis of the powder consumed by the patient allowed to identifying a mixture of 4 different drugs: 2-aminoindane, N-methyl-2-amino-indane, methiopropamine and lidocaine.

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Case description

In recent years, a class of new designer drugs commercialized under the generic name of ''Synthacaines'' have been available to the illicit drug market or deep web. ''Synthacaine'' is the contraction of 2 words ''synthetic'' and ''cocaine'', its name suggests to the consumer this is ''legal cocaine'' developed to mimic the cocaine effect. It is sold both on surface and dark web with a price 3 to 4 lower than cocaine (from ''what illegal drug cost on the dark web consulted on qz.com'').

We report the case of a 30-year-old man admitted to the psychiatry department for acute anxiety crisis accompanied by a sense of imminent danger, impending death and an urge to escape. When he arrived, the patient has been complaining of intense fatigue because he didn't sleep since one week and he had visual hallucinations, like dysmorphophobia responsible for self-harming acts. The patient had

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http://dx.doi.org/10.1016/j.toxac.2017.01.003
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the sensation that his body changed, seeing, for example, his arm turned to evil animals he had to destroy. Effectively, the patient had voluntarily burned himself on repeated occasion during the last week before his admission.

The patient said he took a new drug named ''synthacaine'' he bought on the dark web. The patient could not describe precisely the frequency of his consumption; neither the dose, but he always used the nasal passage. This patient had no medical history before this episode. He was a regular and extensive consumer of numerous stimulant drugs like amphetamines, cocaine and large amount of various drugs bought on the dark web. He doesn't have any mental disease, except short and regressive paranoia with cocaine consumption.

Unfortunately, no biologic samples were available to be analysed, but the patient family allowed us to analyse the substance used.

Material and methods

The Fig. 1 represents the batch of ''synthacaine'' we have analysed. A first black opaque zipped pocket indicate ''research used only'', ''not human consumption, keep away from children and animals'' and all the common Hazardous Substances Information System (HSIS) mentions describing a safe content. A second transparent zipped pocket contain a white/beige colour powder, with the following particulars: ''synthacaine'' and HSIS phrase, different than the black pocket:

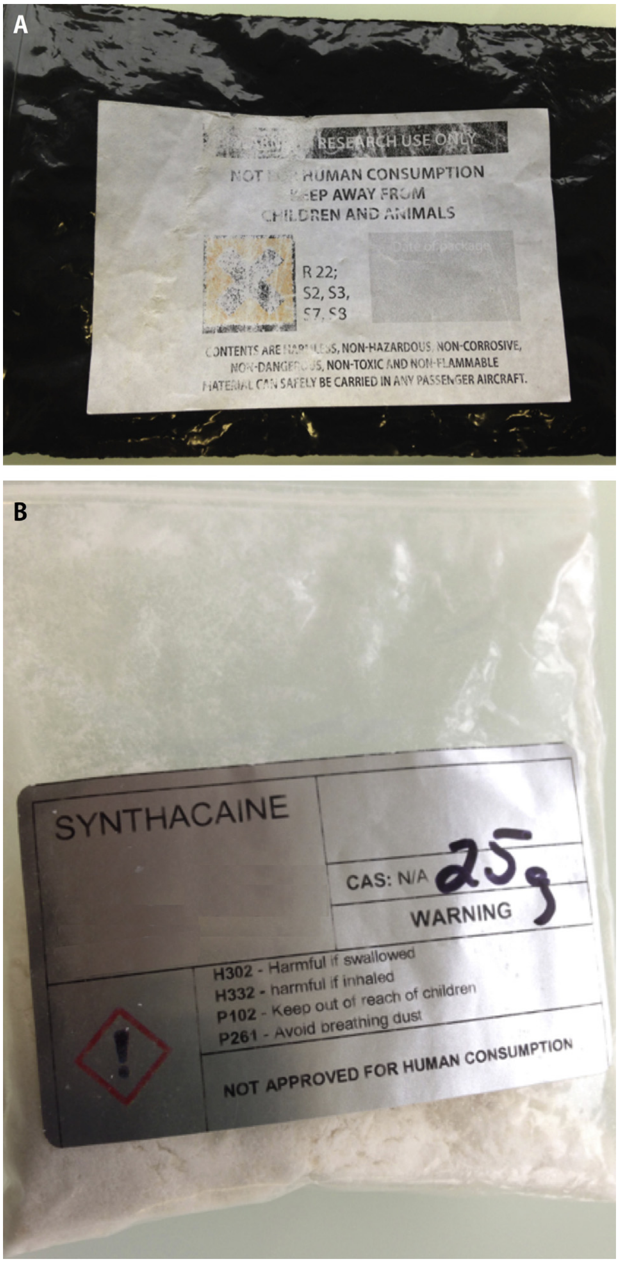
- harmful if swallowed (H302);
- harmful if inhaled (H332);
- keep out of reach of children (P102);
- avoid breathing dust (P261).

Because of few scientific data available concerning ''synthacaine'', various analytics methods were used to identify the active compounds; 1 mg/mL of synthacaine aqueous solutions were used for all the method's investigation. Immunoassays used for urine testing were used as a first screening to clarify the situation. A solution of ''synthacaine'' was analysed with liquid chromatography technique coupled with tandem mass spectrometry (LC-MS/MS): a 10mg/L solution was infused and analysed by General Unknown Screening method (GUS method). A 100 mg/L and 1000 mg/L of aqueous solution of synthacaine were infused both in Gas Chromatography with Mass Spectrometry detection (GC-MS) and Ultra Performance liquid chromatography technique coupled with Diode Array Detector.

Results and discussion

The results obtained by immunoassays methods were synthesised in Table 1. Briefly only amphetamine family was positive by immunoassay technic: amphetamine, ecstasy and methamphetamine were detected in the white powder after aqueous dissolution.

Fig. 2 represents the results obtained by chromatography. These methods allow us to specify the nature of the chemical compounds present in the powder: 2-amino-indane (2AI), N-methyl-2-amino-indane (NM2AI), methiopropamine (MPA) and lidocaine were found with GC-MS, UPLC-DAD and LC-MS/MS methods screening. Chemicals structures of the 4 molecules are represented in Fig. 3.


A
RESEARCH USE ONLY
HUMAN CONSUMPTION
CHILDREN AND ANIMALS
R 22;
S2, S3,
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NON-HAZARDOUS, NON-CORROSIVE,
NON-TOXIC AND NON-FLAMMABLE
SAFELY BE CARRIED IN ANY PASSENGER AIRCRAFT.
B
SYNTHACAINE
CAS: N/A
25g
WARNING
H302 - Harmful if swallowed
H332 - harmful if inhaled
P102 - Keep out of reach of children
P261 - Avoid breathing dust
NOT APPROVED FOR HUMAN CONSUMPTION

Figure 1. Pictures of the synthacaine analysed, the opaque pocket (A) contained the transparent one with the synthacaine powder (B).

The real unknown concerning ''synthacaine'' is the nature of its composition. As we write this column, only 4 publications mentioning the term synthacaine have been identified in Pubmed database [1—4]. The few data available forced us to look for unconventional ways, including the Internet database and consumer's social network.

The composition of ''synthacaine'' revealed by consumer's forum indicates that synthacaine is known to be a mixture: ''...legal substitute of cocaine...'', ''...dimethocaine-like drug mixture...'', ''Official name of

Table 1 Immunoassays detection of the drug tested, cut-off, the method used, and results obtained with a 1 mg/L solution of synthacaine.

Drug (target drug)	''Synthacaine'' Result	Cut-off (ng/mL)	Method
Cocaine (BEG)	Negative	300	EMIT (Xpand Siemens)
Opioids (morphine)	Negative	300	EMIT (Xpand Siemens)
Benzodiazepin (normetazepam)	Negative	200	EMIT (Xpand Siemens)
Barbiturate (secobarbital)	Negative	200	EMIT (Xpand Siemens)
Cannabis (THC)	Negative	50	EMIT (Xpand Siemens)
Methadone	Negative	300	EMIT (Xpand Siemens)
Amphetamine	Positive	1000	EMIT (Xpand Siemens)
Methamphetamine	Positive	1000	Immunochromatography (Narcocheck)
MDMA	Positive	1000	Immunochromatography (Narcocheck)
Buprenorphine	Negative	10	Immunochromatography (Narcocheck)
Fentanyl	Negative	10	Immunochromatography (Narcocheck)
Tramadol	Negative	10	Immunochromatography (Narcocheck)

BEG: Benzoylecgonine; THC: tetrahydrocannabinol; EMIT: enzyme-multiplied immunoassay technique.

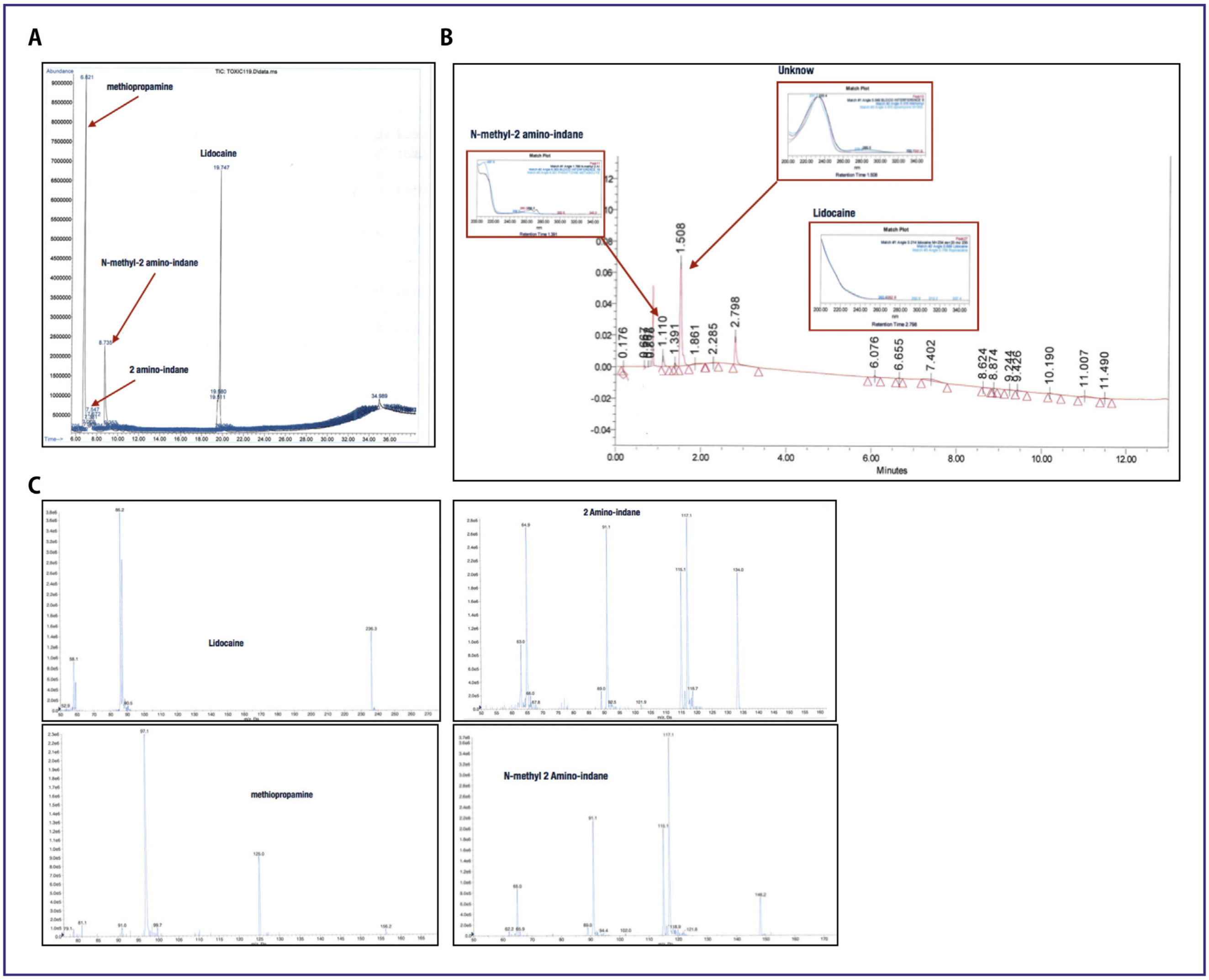

Figure 2. Chromatographic results. A: chromatogram obtained after infusion of aqueous solution in gas chromatography with mass spectrometry detection; B: chromatogram obtained after infusion of aqueous solution in an Ultra Performance liquid chromatography technique coupled with Diode Array Detector; C: chromatogram obtained after infusion of aqueous solution in a high performance liquid chromatography coupled with tandem mass spectrometry.

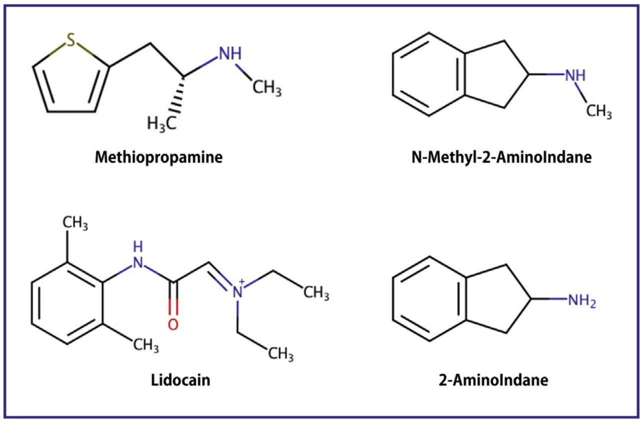

Figure 3. Chemical structures of the four compounds identified in the synthacaine powder analysed.

equal preparation of dimethocain and camfetamine...'', ''...secret-recipe...'', ''There are all different...'', ''...a mixture of methiopropamine/benzocaine or lidocain and sometime 2-aminoindane...'', ''...MPA + b-stock of research clinical...'' (extracted from psychoactive.fr).

According to the sellers on the surfaceweb: ''Synthacaine is actually a sophisticated mixture of several chemicals with complimentary chemical properties'', ''The recently banned product of MPA has now been replaced with the very popular 3-FPM'' ...so instead of dimethocaine and other similar chemicals used by some vendors, we use our own blend of ''-caine'' (extracted from euchemicals.com).

According to scientific research in 2016, a French team described an acute intoxication with methiopropamine, in 2015; Cumba et al. published identification of a synthacaine's batch containing a mixture of methiopropamine and 2-aminoindane, whereas in 2014, Lonati et al. published a surprising synthacaine batch containing a synthetic cannabinoids MAM-2201 [2—4]. The specialised Welsh Emerging Drugs and Identification of Novel Substance published via its website the composition of various batch of synthacaine they have analysed; the main compounds that were found were lidocaine, procaine and methiopropamine (extracted from wedinos.org). The main associations were a local anaesthetic (lidocaine or procaine) + methiopropamine occasionally associated with 2-aminoindane [5].

Conclusion

''Synthacaines'' are a new marketed class of psychoactive substance, developed to mimic the cocaine effect. ''Synthacaines'' are sold on specialised website as ''legal cocaine'' and are available for a price approximately 4 times lower than cocaine. ''Synthacaines'' are always a combination of substance in which precise molecules compositions varied from seller to other. They usually contain an association of local anaesthetic and amphetamine-like molecules [5]. The effect varies from one consumer to another, but this pharmacologic variability is probably more the consequence of the composition's difference than a real inter-individual variability. In our case, 3 psychoactive molecules were identified. Considering the MPA, users report similar effects to other stimulants such as MDMA (ecstasy), amphetamine and cocaine: stimulation, alertness and an increase of energy and focus; with adverse effects reported by users including tachycardia, anxiety, panic attacks, sweating, headaches, nausea, difficulty breathing, vomiting, difficulty urinating and sexual dysfunction. Because of an amphetamine-like core structure, 2AI and NMAI are expected to interact with the norepinephrine, dopamine, and serotonin pathway as with their respective transporters by blocking or releasing monoamines[6,7]. Based on their higher potencies at serotonin transporter than dopamine transporter, 2AI and NM2AI derivative could be more empathogen psychoactivity, than they have potential for abuse and addiction [8,9].

For the aminoindane derivatives, it seems that the drug's overall behavioural profile would be related to its serotonergic effects, because of their empathogenic and entactogenic effets. MDAI induce serotonine release mainly via serotonin transporter inhibition [9]. Lidocaine is not a NPS but a local anaesthetic. Lidocaine, as other substance like sugar, caffeine, phenacetine, levamizole, is frequently used to cut or adulterate illicit drug [10]. Nevertheless, medical use of lidocaine show that the addition of lidocaine appeared to reduce the discomfort of intranasal unpleasant and painful citrate solution in adult volunteers [11]. Moreover, lidocaine is equally used to enhance or mimic the effects of illicit drugs [10].

The few trip reports available on the consumer forum and self-reported effect available on Wedinos indicate stimulating, significant energy, increased confidence, increased senses, increased stamina, increased libido effect but seem equally to be entactogenic and empathogenic substances (extracted from wedinos.org). Hallucinations caused by the ''synthacaine'' mixture used by our patient were a rare example of bad trip, one of the first described at this time.

Disclosure of interest

The authors declare that they have no competing interest.

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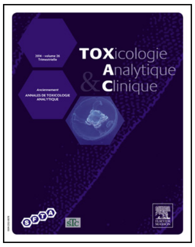

CASE REPORT

LSD instead of 25I-NBOMe: The revival of LSD? A case report

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Received 26 October 2016; received in revised form 20 December 2016; accepted 21 December 2016

KEYWORDS
NPS;
NBOMe;
LSD;
Screening

Summary
Observation. — We report a case of a 25-year-old man crushed by a train while he was returning from a rave party. The consumption of 25I-NBOMe was rapidly evoked by people who had participated at the party.
Materials and methods. — In this context, the post-mortem toxicological expertise was completed by a broad screening using high-resolution mass spectrometry detection (LC-HRMS). Three hundred NPS and metabolites (including 25B, 25C and 25I-NBOMes) can be found with this method. A targeted screening in MRM mode was also performed on a smaller number of hallucinogens.
Results. — The toxicological analyses were performed on blood and urine samples. Amphetamines, cocaine, cannabinoids and opiates were not detected. Ethanol was measured at 0.71 g/L and 1.59 g/L in blood and urine samples, respectively. The screening by LC-HRMS did not reveal the presence of NPS, including NBOMes. The targeted screening in MRM mode revealed the presence of LSD and its metabolite, the 2-oxo-3-hydroxy-LSD. LSD was quantified at 0.2 ng/mL in blood.

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http://dx.doi.org/10.1016/j.toxac.2016.12.007
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Conclusion. — This case alerts on the frequent confusion between NBOMes and LSD and on the renewed interest for LSD due to the popularity of NBOMes. This case therefore encourages the prudence and research of all hallucinogenic substances, even when the context is evocative.

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Introduction

New psychoactive substances (NPS) are sold as ''legal highs'' or ''research chemicals'' and can be categorized in five major groups upon their parent compounds according to Baumeister et al. [1]: cannabinoids, stimulants similar to amphetamines or cocaine, benzodiazepines, dissociatives similar to ketamine and hallucinogens similar to LSD or psilocybin. They represent currently a major public health problem. Thus, several problems related to the use of NPS emerge: the rapid synthesis of new derivatives to circumvent the drug laws, the ignorance of the toxicity of the new products marketed, the confusion between the product supposedly consumed and the product really consumed and mixtures [2]. Among the new emerging NPS, NBOMes, proposed as a legal alternative to the hallucinogens (LSD, phenethylamines and tryptamines), occupy an increasingly important place. NBOMes, known in the street under various appellations (boom, pandora, nbomb...), are sold on the Internet as blotter paper or drops, not destined for human consumption [3]. Their distribution is facilitated by their attractive price, between 7 and 10 $. Pharmacologically, NBOMes are highly potent agonists of serotonin 5-HT2A receptors [3] and are active at low doses (between 500 and 800 μg for the 25I-NBOME for example). Structurally, they are derivatives of phenethylamine 2C-X (phenethylamines on which two methoxy groups on positions 2 and 5 and a halogen on position 4 were added to increase the hallucinogenic effect). The addition of the 2-methoxy-benzyl group (called BOMe) is used to protect the amine group and potentiate the affinity for the 5-HT2A receptor [4]. NBOMes can be used through several ways. The sublingual route is the most widely used because of its better bioavailability. The nasal route can also be used for drops. Other routes of administration have been reported more anecdotally (injectable [IV or IM], pulmonary, vaginal, rectal). As with other hallucinogens, recreational users are looking for a physical and mental stimulation, as well as psychedelic effects that can occur by a disturbance of perception of the environment, a change of time, visual and auditory hallucinations. The duration and intensity of the effects depend on the molecule, dose and route of administration. Thus, for the 25C-NBOMe, hallucinogenic effects have been reported after the consumption of 200 to 1000 μg sublingually or of 50 to 500 μg after inhalation [5]. NBOMes do not share the favorable toxicity profile of conventional hallucinogens [6]. Thus, with the NBOMes, the consumer is exposed to a serotoninergic syndrome (nausea, vomiting, confusion, agitation, tremors, hyperthermia, sweating, hypertension, tachycardia, mydriasis, metabolic acidosis, rhabdomyolysis, renal insufficiency, disseminated intravascular coagulation) [7]. Recently, several cases of death linked to NBOMe consumption have been described in the literature [8—11]. However, the popularity of NBOMes should not make us forget the use of conventional hallucinogens. We present here a case of death after a supposed NBOMe intake which turned out to be an accidental death linked to LSD consumption.

Observation

We report the case of a 25-year-old man who died after being crushed by a train. According to the information gathered on the spot by the police, the young man had participated at a rave party not far from the scene of the accident. The consumption of 25I-NBOMe was then rapidly evoked by people who also participated at the party.

Materials and methods

The initial toxicological analysis of post-mortem biological fluids (peripheral blood and urine) was performed by several methods: high performance liquid chromatography coupled with diode array detector (HPLC/DAD) using an Alliance system (Water^{TM}) for screening and drug monitoring, gas chromatography coupled with mass spectrometry (GC/MS) using a QP2010 system (Shimadzu^{TM}) for screening and drug identification (cocaine, opiates, cannabinoids and amphetamines), gas chromatography with flame ionization detector (GC/FID) (ThermoFisher Scientific^{TM}) for alcohols.

In this context, the post-mortem toxicological expertise was completed by a LC-HRMS screening method (M1) using a Xevo-G2 QTOF-XS system (Waters^{TM}) [12]. A targeted LC-MS/MS method (M2) using a XEVO TQS system (Waters^{TM}) was also performed on a smaller number of hallucinogens. Briefly, for M1, 400 μL of sulfosalicylic acid (3%) was added to 100 μL of sample. After on-line extraction using OASIS$^{®}$ HLB column (2.1 × 30 mm, 20 μm) (Waters^{TM}), separation was performed on a Acquity^{TM} UPLC HSS C18 column (2.1 × 150 mm, 1.8 μm) (Waters^{TM}) and detection occurred in MSe (ESI+) mode. Data process was performed using ChromaLynx^{TM}, TargetLynx^{TM}, MassFragment^{TM} and MetaboLynx^{TM} associated softwares (Waters^{TM}) using a homemade database of more than 1.400 substances including nearly 300 NPS or metabolites (including 25B, 25C and

25I-NBOMes and metabolites). For NBOMes, Limit of Detection (LOD) is of the order of 1 ng/mL with this method. For LC-MS/MS method (M2), 100 µL of sample was deproteinized (300 µL methanol), diluted (100 µL of ammonium formate/0.1% formic acid buffer), and analyzed after separation by an Acquity™ UPLC BEH C18 column (Waters™). Detection was achieved in multiple reaction-monitoring (MRM) mode (ESI+) for 222 NPS or metabolites.

The determination of LSD concentration was performed according to a validated method on a TSQTM Vantage triple-quadripole mass spectrometer equipped with an electrospray ionization source set in positive mode. Chromatographic separation was performed on a Hypersil Gold PFP column (ThermoFisher Scientific™, 1.9 µm, 100 × 2.1 mm). Elution was in isocratic mode with a mobile phase composed by a mixture of ammonium formiate 2 mM with formic acid 0.1% and acetonitrile (50/50, v/v) pumped at 0.3 mL/min. To exactly 1000 µL of sample (calibration standards, quality control or patient samples) were added 10 µL of internal standard (LSD-D3 0.1 mg/L), 500 µL of carbonate buffer (pH = 9.7) and 4 mL of diethylether-toluene (60/40, v/v). After centrifugation, the upper organic layer was evaporated to dryness. After evaporation of the organic phase and reconstitution with 100 µL of the mobile phase, 10 µL were injected into the system. Data were collected in selected reaction monitoring (SRM) mode, with three *m*/*z* transitions for LSD and one for IS. LSD was monitored at 324.2 → 223.15 (used for quantification) (Fig. 1B), 324.2 → 197.12 (Fig. 1C) and 324.2 → 208.11 (Fig. 1D). LSD-D3 was monitored at 327.3 → 226.3 (Fig. 1A). Limit of Quantification (LOQ) of LSD was determined at 0.125 ng/mL.

Results

Amphetamines, cocaine, cannabinoids and opiates were not detected. Ethanol was measured at 0.71 g/L and 1.59 g/L in blood and urine samples, respectively. The LC-HRMS screening did not reveal the presence of NBOMes or their metabolites in blood and urine. The targeted screening in MRM mode revealed the presence of LSD and its metabolite, the 2-oxo-3-hydroxy-LSD in blood and urine. LSD was quantified at 0.2 ng/mL in blood (Fig. 1). The volume of urine was not sufficient to carry out an assay in this matrix.

Discussion

The context and the notion of a potential 25I-NBOMe intake led to the achievement of a broad toxicological screening searching for NPS and their metabolites. The detection of NPS and, in particular, of 25I-NBOMe, which had been mentioned by people who had participated at the party, was negative. However, the non-detection of 25I-NBOMe in the blood and urine samples from the victim does not completely exclude the intake. Indeed, NBOMes are active at very low doses and concentrations are low, less than 1 ng/mL in blood and less than 3 ng/mL in urine, as it was illustrated in several documented cases of intoxication for which detection and measurement of NBOMe concentrations were carried out [9,13—15] (Table 1). The targeted screening of hallucinogens revealed the presence of LSD and its metabolite, the 2-oxo-3-hydroxy-LSD, in blood and urine samples. The detection of LSD and its metabolite is consistent with the context. In France, the use of LSD in the general population is very rare (0.3% of people aged from 18 to 64 years old in 2014). The proportion of users in the young population is greater, but still low (1% of the 18—25 years old) [16]. LSD appears especially available in the festive space linked to electronic music, as reported in the network ''Trends recent and new drugs'' (TREND) of the French Observatory of Drugs and Drug Addiction (OFDT) [17]. It seems that its image is particularly good in this space. Within this festive space, the levels of LSD experimentation could reach 51% and up to 71% in alternative environments [17]. The availability of LSD seems to vary from year to year, but prices are stable (average price of one dose: 10 €). LSD is mostly sold as colorful blotting paper, in liquid form or more rarely as microtip. Moreover, the effects of LSD have been well described. The psychological effects appear after a dose of 75—200 µg and are marked by a feeling of euphoria and hallucinations with distortion of time and space [18]. Rarely, bad trip experiences may also

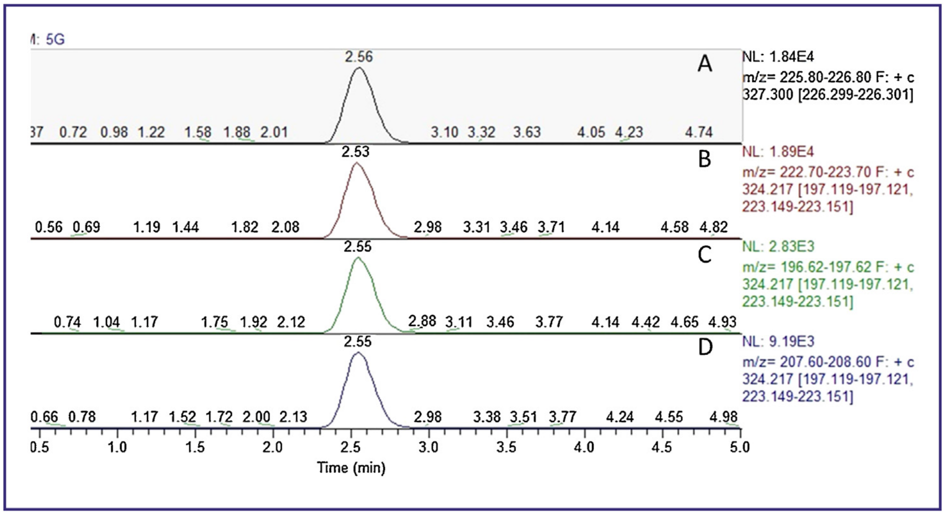

Figure 1. Chromatograms of LSD (B to D) and LSD-D3 (A) in blood sample.

Table 1 NBOMe concentrations in documented cases of poisoning.

References	Drug	Serum concentrations (pg/mL)	Urine concentrations (pg/mL)	Clinical signs
Poklis et al. [13]	25B-NBOMe	180	1900	Agitation, fever, hypertension, tachycardia, rhabdomyolysis
Poklis et al. [9]	25I-NBOMe	405	2800	Death after defenestration
Suzuki et al. [14]	25I-NBOMe	34	—	Visual hallucinations, suicide attempt, hypertension and mydriasis
Rose et al. [15]	25I-NBOMe	760	—	Agitation, aggression, hallucinations, tachycardia, hypertension, mydriasis

occur with mood swings (from euphoria to extreme anxiety) and phenomena of flashback [19]. The described somatic effects are related to a sympathetic stimulation of the autonomic nervous system (mydriasis, moderate increase of heart rate and blood pressure) and a parasympathetic stimulation (sweating, salivation and nausea). These effects appear after 30—40 min with a maximum between 1.5 and 2.5 h. Hallucinogenic effects of LSD are mainly explained by an agonist effect on 5-HT2A receptors, although an agonist effect on 5-HT1 and 5-HT2 receptors has also been described [18]. Apart from bad trip experiments and suicide attempts, organ toxicity is rarely mentioned for LSD. Only a few cases of convulsion or toxic coma with respiratory depression have been reported in the literature [20,21].

The LSD concentration measured at 0.2 ng/mL in the blood of the victim is consistent with those described in the literature for usual dosage of LSD. Thus, the plasma concentration following IV administration of 2 μg/kg of LSD is in the order of 1 ng/mL 8 hours after administration [22]. The measurement of the 2-oxo-3-hydroxy-LSD concentrations, although of interest, could not be carried out with our method.

On the illicit market, confusion between LSD and NBOMes is relatively frequent [14,23]. In a review of 20 cases of NBOMe poisoning published by Suzuki et al., 20% of users thought they consumed LSD instead of NBOMes [24]. The outcome of those poisonings was fatal in 2 cases. There are few documented deaths due to LSD overdose [25,26]. In contrast, several cases of death related to a serotoninergic syndrome following NBOMe consumption have been reported since their appearance on the market [27].

Conclusion

The emergence of NPS is currently a major public health problem. Analytical toxicology laboratories must develop innovative screening methods to identify them when the context is evocative. This involves very important work to constantly update screening libraries and the use of very sensitive methods, such as LC-HRMS, since these molecules are active at very low doses. However, this should not prevent the search for the main reference drugs even when the context is evocative. We illustrate here a case of death after a supposed 25I-NBOMe intake which turned out to be a death linked to LSD consumption. This case alerts on the frequent confusion between NBOMes and LSD and on the renewed interest for LSD due to the popularity of NBOMes. This case therefore encourages the prudence and research of all hallucinogenic substances, even when the context is evocative.

Disclosure of interest

The authors declare that they have no competing interest.

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LETTER TO THE EDITOR

The comeback of Ecstasy: New designs and increased MDMA content

To the editor

Ecstasy (XTC), the tablet form of 3,4-methylenedioxymethamphetamine (MDMA), was popular, regarding its wide use and abuse, in the 1990s in the clubs and discotheques and even more in the techno free parties and underground nightlife events. Indeed, recreational XTC use is historically highly linked to the underground techno subculture, which was born in UK in the late 1980s during the second “summer of love”, to such an extent that this movement was called “Ecstasy Culture”. In the 2000s, changes in laws and regulations have altered the organization of festive techno space and, subsequently, the use of psychoactive drugs within it [1]. In 2008, the Cambodian authorities launched a large-scale operation to destroy many Sassafras cultivation. As the safrole, one of the main precursors for the MDMA synthesis, comes from this shrub, these destructions led to an international shortage of MDMA. Consequently, pills sold for ecstasy often contained other molecules which sometimes led to side adverse effects. More and more, XTC gets a bad reputation, considered as a scam, and his popularity decreased. The real XTC became rarer and after the shortage, MDMA “crystal/rock” forms emerged on the market and were preferred by users owing to assessments of their high-purity [2]. This period also coincides with the gradual emergence of new psychoactive substances (NPS). “Research chemicals”, “bath salts”, or “legal highs” are other terms used for NPS which are usually sold via Internet websites [3]. Marketed in many different ways and forms, NPS use can be observed among many different user groups. Today, the global market for NPS continues to expand and a growing number of NPS is reported each year throughout the world although significant differences between countries and regions [4,5].

Since 2011, XTC has made a comeback in festive settings [6]. Today, XTC has evolved in order to increase attractiveness for young people including 3D-shapes, bright colors, and updated logos (i.e. Nespresso, Bentley, Bugatti, Durex, Nintendo...). Moreover, increases of the masses of the tablet and the MDMA content per tablet have been reported [7]. For instance, since 2014, 41 % of the alert messages delivered by Eurotox in Belgium have been related to XTC. Among these data coming from Belgium, Netherlands, Spain and United Kingdom, 73 % reported a risk associated with high dosages of MDMA and 27 % concerned tablets containing NPS instead of MDMA [5,8].

According to this evolution and to associated health risks, the French Monitoring Centre for Drugs and Drug Addiction (Observatoire français des drogues et toxicomanies [OFDT]) spearheaded a survey in order to evaluate the composition of XTC that is currently circulating in France.

More than one hundred XTC were collected from users between December 2014 and May 2015. Twenty-one tablets have been received and analyzed in our laboratory. Each tablet was photographed, described, weighted and measured. After thin manual grinding using a mortar, one 10-mg-aliquot of each sample was solubilized in 1 mL of methanol, sonicated for 5 minutes, and, subsequently, 1:10,000 diluted in methanol: 100 μL of the obtained solution were evaporated to dryness using nitrogen after internal standards addition (methyl-clonazepam and β-OH-ethyltheophyllin). The obtained residue was reconstituted in 100 μL of the mobile phase and then analyzed using liquid chromatography with high-resolution mass spectrometry detection (LC-HRMS) with a UPLC-Xevo G2 QTOF (Waters, Manchester, UK) system. The chromatographic separation was performed using an Acquity TM HSS C18 column (1.8 μm, 2.1 × 150 mm) (Waters) with an oven temperature of 50 °C and a mobile phase gradient consisting in ammonium formate buffer pH 3/acetonitrile in 0.1 % formic acid. Ionization and acquisition were performed in positive electrospray mode and using the MSE mode, respectively. The LC-HRMS device is controlled by MassLynx™ 4.1 software (Waters). Data process and quantitation were performed using ChromaLynx™, TargetLynx™ associated softwares (Waters) using a homemade database of more than 1400 substances including MDMA [9].

The 21 XTC analyzed samples were visually high quality tablets with various shapes and bright colors. Logos can effectively be considered as attractive, including actual trademarks: Nespresso, Chupa Chups, UPS, Louis Vuitton, Rolls Royce, Superman, Mitsubishi, Spiderman... Seventeen tablets with an average weight of 321 mg (ranging from 196 to 514 mg) contained an average quantity of MDMA of 145 mg (ranging from 69 to 279 mg) what therefore means that the tablets contained 46 % of this substance (ranging from 22 to 72 %). No MDMA was found in 4 tablets: one contained meta-chlorophenylpiperazine (mCPP), two contained only acetaminophen, and no active substance was found in the last one.

http://dx.doi.org/10.1016/j.toxac.2016.09.003

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The analysis results of this sampling were in accordance with those obtained by the other laboratories involved in this survey and with the current observations made by the other European countries, in terms of marketing efforts and purity. Moreover, those results underlines the dramatically increase of the average amount of MDMA per tablet during the two past decades. In the 2000s, the value was around 50—60 mg per tablet, [10—12] whereas today it is more often around 145 mg and 125 mg per tablet respectively in the French and the European market. The increase of the MDMA amount is logically linked to the rise of the tablets weight but it has also been observed a slight increase of the concentrations since the beginning of the 21st century. Considering that there is a persistent number of tablets without MDMA or containing another substance as a NPS (4/21), this survey and the elements previously documented [5,13,14] show the great variability and the dynamism of the XTC market. By the way, they also interrogate how drug users adapt their habits considering those changes.

Users often pretend that they can know the content and the purity of their tablets by comparing their macroscopic characteristics. That is why it is important to remind them that on the one hand, a batch of tablets has a short life on the market. On the other hand, ingredient proportions or cooking manners are likely to change from one production cycle to another. Even if two tablets are visually strongly similar, their compositions could be totally different. Their weight, the presence or not of scoring line are not reliable. The only way to know the ecstasy content is to analyze it. It is undoubtedly even more important to ease the access at such analyzing services during nightlife events, where users are likely to purchase and consume drugs.

Disclosure of interest

The authors declare that they have no competing interest.

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Received 5 August 2016;
received in revised form 16 September 2016;
accepted 17 September 2016

Available online 20 October 2016